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The purification and concentration of hog cholera virus.

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THE PURIFICATION AND CONCENTRATION OF HOG CHOLERA VIRUS

by

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DEDICATION

to Lois

INTRODUCTION

Hog cholera is a highly contagious virus disease of swine which has persisted in the United States since about 1840. The disease is characterized by sudden onset in susceptible swine populations, followed by a high morbidity and mortality incidence. Although it is experimentally possible to propagate hog cholera virus in certain other animal species, wild and domestic pigs are its only known natural hosts. Historically, hog cholera epidemics of disastrous intensity were periodically observed in the United States, particularly in areas where swine husbandry figured significantly in the regional livestock industry. Hog cholera continues to be a worldwide disease problem of swine in most countries where swine are raised in substantial numbers.

Even though the incidence of hog cholera has generally declined in the United States since about 1950, persistence of the disease seriously handicaps export trade in pork products and contributes significantly to the total economic losses in our swine industry. A steadily increasing use of vaccines, and application of rigorous diagnostic and control techniques undoubtedly accounts for the current low incidence of hog cholera in the United States. However, the average annual cost of controlling hog cholera in this country remains at about $40,000,000 (1). Between 1940 and 1960 this figure approached one billion dollars or the approximate present

value of our entire swine industry. The magnitude and persistence of economic losses due to hog cholera resulted in the current Federal program for eradication of this disease in the United States.

Because most research with hog cholera virus has been devoted to the histo-pathology of the disease, development and improvement of various diagnostic techniques, vaccines and immunization procedures, very little is known about the chemical and physical properties of hog cholera virus. As a result, little progress is evident relative to an objective serologic, immunologic, and taxonomic characterization of the viral agent. Probably the most important single feature responsible for such slow progress is the fact that few cell types will propagate the virus in culture, and no direct in vitro cytopathology has been established for hog cholera virus. Thus, until recent times hog cholera virus infectivity assays were obtained using large numbers of swine; a rather ponderous, uneconomical procedure often difficult to control and interpret. The first in vitro assay procedures for hog cholera virus and its antibody involve modification of the cytopathic effects due to Newcastle virus and interference with bovine diarrhea virus.

Recent development of a fluorescent-antibody plaque assay for hog cholera virus provides a direct, reliable in vitro method for accurate, quantitative infectivity measurements. With this procedure it is possible to assay hog cholera

virus by enumerating infected cell plaques stained with fluorescein-labeled specific antibody. Not only is it possible now to assay infective virus for diagnostic and other laboratory routines, but the availability of such a procedure is pre-requisite for any systematic study bearing on the physical-chemical properties of hog cholera virus.

An adequate body of data regarding the physical-chemical properties of a virus agent often leads directly to a rapid accumulation of practical information related to the serologic and immunologic properties of that virus. Since concentration of the agent concomitant with relative freedom from extraneous materials is usually pre-requisite to chemical-physical analyses, a most useful early step in this context is development of a practical purification-concentration procedure. The first objective of this study was to define optimal conditions for the partial purification of HCV using adsorption chromatography on magnetic ferric oxide. The second objective was to achieve a final degree of purification and concentration by ultra-centrifugation in (cesium chloride) density gradients. As a practical application for these procedures, an attempt was made to obtain an electron-micrograph of hog cholera virus.

LITERATURE REVIEW

A systematic search of literature regarding the characterization of hog cholera virus yields relatively few reports containing pertinent data. Most of this data was published subsequent to 1961 when the first *in vitro* assay for HCV was described (2). Prior to 1961, the difficulty in conducting infectivity assays is reflected by a near absence of data bearing on the physical-chemical properties of HCV.

The viral nature of hog cholera was established in 1903 when De Schweinitz and Dorset (3) demonstrated that the infectious agent could pass through fine porcelain filters which excluded all bacteria. In 1929, Kernkamp (4) described filtration experiments which indicated the size of HCV was less than 35 mμ. In 1949 Bachrach described similar filtration experiments which indicated that the size of HCV was between 17 and 25 mμ (5). Boynton *et al.* (6) were able to obtain the first electron-micrograph of hog cholera material in 1948. Although their photos were relatively crude when compared to results obtained with present-day equipment, the photos do show objects which may be virus particles. Using extracts of spleen tissue from pigs infected with HCV, Bachrach also obtained electron micrographs of chromium shadowed objects which resembled virus particles (5). However, similar particles were observed with the electron microscope in specimens prepared from normal pig spleens. Similarly, in 1951, Reagan *et al.* (7) published

electron-micrographs showing objects with a mean diameter of 27 mμ which they obtained from infectious pig serum.

In a more recent electron microscope study, Ageev (8a) reports seeing particles 20 and 40 mμ in diameter on erythrocyte membranes from blood of pigs infected with HCV. Using ultra-filters, Pehl and Gralheer (8b), determined a size range of 15-25 mμ for HCV. Wilner (9) summarizes these data by giving a range of 15 to 44 mμ for HCV. Even with such variation in the data pertaining to size, it is evident that HCV is a rather small animal virus.

In 1962, Dinter (10) described the first systematic study to determine certain characteristics of HCV using taxonomic criteria such as those suggested by Wilner (9). Dinter's results indicate that HCV is a RNA-containing virus, sensitive to ether, chloroform and sodium desoxycholate, moderately sensitive to trypsin and not stabilized to heat by molar magnesium chloride. Loan (11) confirmed Dinter's work with respect to nucleic acid type and lipid solvent sensitivity. McKissick (12) further confirmed the sensitivity of HCV to lipid solvents by repeating Dinter's experiments with ether, chloroform and desoxycholate.

Although these data are important taxonomic points, a state of confusion is evident in the literature regarding the relationship of HCV to certain major groups of animal viruses. Dinter arranged his data to show a relationship between HCV

and bovine diarrhea virus (BVD) and further suggests that both agents may be related to the group B Arboviruses due to the nature of their trypsin sensitivity. In contrast, Ditchfield and Doane (13) suggest that BVD be considered a myxovirus due to its morphology as seen with the electron microscope. They further suggest that HCV may also be a myxovirus by virtue of its relationship to BVD with respect to nucleic acid type, ether sensitivity and the presence of a soluble antigen common to both agents (14). The concept of HCV as a myxovirus is a rather vulnerable one since HCV would occupy a unique position with respect to size, being far smaller than the currently accepted range of 80-200 mμ for the myxoviruses (15). Furthermore, there is no evidence that HCV will agglutinate erythrocytes, induce hemadsorption or otherwise show affinity for mucoprotein substances, the latter being a fundamental characteristic of the myxoviruses (9). Classification as a group B Arbovirus probably is less risky but the requirement for an insect vector, so far as is known, is not fulfilled with HCV. Wilner (9), therefore, hesitantly lists HCV as a "possible" myxovirus while most recently Packer (16), due to lack of sufficient taxonomic data, places HCV in a list of unclassified animal viruses. A decision concerning an appropriate position for HCV within the major groups of animal viruses, seems now to depend more on morphological data obtained with electron microscope studies of concentrated, purified virus.

In recent years cell culture techniques have been widely applied to the investigation of a great variety of viral agents, particularly those which cause direct, readily observed cytopathic effects in cell cultures. Unfortunately, such techniques have had only limited application in hog cholera research since characteristic cytopathology has not yet been established for HCV. Propagation of HCV in either primary or established cell lines from swine tissues has generally been successful but, until recent years, applied only to vaccine studies.

The first widely used *in vitro* assay for HCV was described by Kumagai *et al.* (2) in 1961. This method, designated END (exaltation of Newcastle disease), originated from the finding that HCV, by itself a noncytopathogenic virus, enhances cytopathic changes in swine testis cells subsequently infected with the Miyadera strain of Newcastle disease virus (NDV). Briefly, the test involves inoculation of primary swine testis cell cultures with various dilutions of HCV. After four days of incubation each culture is challenged with a high dose of NDV and cytopathic changes are noted following an additional three-day incubation period. Cytopathic effect is considered as evidence of the presence of infective HCV. The same workers later demonstrated that END could be blocked by hog cholera antibody and described a virus neutralization procedure for HCV using END (17). In 1962, Nishimura *et al.*, described a similar *in vitro* assay procedure for HCV involving use of the

Sato strain of NDV but which resulted in exaltation of hemagglutination and inhibition of cytopathic effects (HEIC) due to NDV (18). Obviously, when considering use of either the HEIC or END methods, the researcher must carefully select the proper NDV strain and determine its behavior with respect to the strain of HCV under investigation.

Although these methods provide a means for conducting more extensive and expedient hog cholera research, they are not without disadvantages. Both procedures require use of an additional virus agent and involve indirect methods subject to the eccentricities of cell culture techniques used at various laboratories. Also, considerable time may elapse before results can be obtained; 9-11 days for HEIC and 7-9 days with the END method. However, each method is a distinct blessing to those concerned with hog cholera research and both have been instrumental in providing much of the recent data regarding HCV.

Another possible method for *in vitro* HCV assays was indicated in a recent report by Malmquist, *et al.* (19). These workers adapted a cytopathogenic strain of BVD to both primary and an established cell line of swine kidney cells and noted that cytopathic effects of BVD were abolished by prior infection with HCV. They also noted that the interference was dose-dependent with respect to HCV and infection of cells with HCV was required before interference was observed. There is yet no evidence in the literature that this procedure has been

applied to hog cholera research. Its disadvantages would be similar to those mentioned for the END and HEIC procedures.

Literature concerning cytopathology produced by HCV first appeared in a 1956 publication by Gustafson and Pomerat (20). Microscopic cytopathic changes were observed in lung and spleen cell cultures derived from a pig infected with HCV, and in cell cultures from pig embryo lymph nodes which were infected in vitro (21). Gillespie et al. (22) observed cytopathic effects with a virus isolated from a pig chronically infected with HCV. This virus was sub-culturable in primary pig kidney cell cultures and was designated as cytopathogenic HCV, strain A. Cytopathic effects of strain A were neutralizable by HCV antibody, a feature later (23) used in serological studies relating HCV and BVD. Bass and Ray examined Gillespie's strain A using acridine-orange fluorescence methods and described the cytopathic changes observed in embryonic pig kidney cells (24). Segre obtained cytopathic effects with HCV in cell cultures maintained under high oxygen tension (25). Mayr and Mahnel considered that the cytopathogenicity of HCV was dependent on very special cultural conditions and produced electron micrographs of virus particles propagated with their methods (26). A striking feature of their second report is the presence of adenovirus particles contaminating cell cultured preparations originally thought to contain only HCV. Thus, it may be that contaminating adenoviruses are responsible for cytopathic effects seen with other cytopatho-

genic strains of HCV. In a recent study directed toward resolving this question, Bodon has shown rather conclusively that with each of the seven "cytopathogenic" strains of HCV he observed, cytopathology was due to contaminating adenoviruses (27). He concludes his report by stating, ".....that in every case when HC virus exhibits a cytopathic action, a contaminant virus should be considered and checked by thorough cytochemical examination." In view of these findings, caution must be exercised when interpreting results of hog cholera research involving the alleged cytopathogenic strains of HCV.

Application of fluorescent antibody techniques to detection of HCV in cell cultures has been described by Solorzano (28) and by Mengeling et al. (29) and was subsequently used by Carbrey et al. (30) and by Mengeling (31) to estimate relative concentrations of HCV in tissues from infected pigs. Mengeling later modified the procedure to obtain a high degree of accuracy for HCV infectivity titrations based on enumeration of infected cell plaques stained with homologous fluorescent antibody (32). With this procedure, reproducible infectivity assays are obtained within 48 hours after dilutions of HCV are inoculated into Leighton cell-culture tubes containing HCV-susceptible cell monolayers.

Major disadvantages of the fluorescent-antibody plaque assay (FAPA) procedure concern its requirement for ultraviolet light microscopy and the preparation of fluorescein-labeled specific antibody for HCV. A minor disadvantage

concerns the fact that fluorescein-labeled hog cholera antibody may also bind to soluble antigen produced in cells infected with BVD (33). Therefore, appropriate precaution in laboratory procedures should be exercised to avoid such nonspecific reactions. It is also notable that performance of the test in primary cell cultures has not yet been explored but is limited to use with an established cell line derived from pig kidneys (32). However, the advantages of speed and accuracy obtained with the FAPA procedure easily outweigh its disadvantages and place it in a position superior to other *in vitro* assays for HCV. Consequently, the FAPA procedure as described by Mengeling, was used for all HCV infectivity titrations in the present study (32).

Application of concentration and purification methods to studies concerning HCV has, until recently, been frustrated by the absence of a suitable *in vitro* infectivity assay procedure. Boynton *et al.*, Bachrach, Reagan *et al.*, and Mayr and Mahnel used combinations of methanol precipitation and differential centrifugation prior to electron microscopy (6, 5, 7, 26). However, such methods, with the exception of results obtained by Mayr and Mahnel, failed to achieve a purification sufficient to show discrete virus particles.

Kulesko *et al.* described a concentration-purification procedure for HCV involving homogenization of virus-laden tissues with a fluorocarbon organic solvent (34). A successful vaccine was produced using this method but the authors

indicate that further study was required before the procedure could be defined. Similar results were reported by Bychkov who also used a fluorocarbon to purify and concentrate HCV (35).

Using a DEAE-cellulose chromatographic procedure, Pirtle successfully partitioned HCV and its soluble antigen (36). Although the procedure was developed to study the isolated soluble antigen of HCV, good yields of infective virus were obtained. While it is evident that HCV purification may be obtained with such a procedure, a practical application would require an additional step for concentration of HCV.

An important set of data which suggest a purification and concentration procedure for HCV was recently reported by Horzinek who described the stability and buoyant density of HCV in cesium chloride solutions (37). After demonstrating that HCV was stable for several hours in cesium chloride, Horzinek used isopycnic density centrifugation to determine that the buoyant density of HCV ranged from 1.16 to 1.18 in cesium chloride. These data are similar to data obtained by Crawford in studies concerning the buoyant density of Rous sarcoma virus (RSV) in rubidium chloride (38). However, Crawford concentrated RSV in a preliminary step by ultracentrifugal sedimentation onto a dense layer of rubidium chloride. Purification of RSV was subsequently obtained by isopycnic density centrifugation in rubidium chloride. The methods successfully used by Crawford suggested that similar

results might be obtained with HCV if cesium chloride were used to obtain the densities indicated by Horzinek's data.

Another interesting method which yields partially purified preparations of myxoviruses has been described by Warren et al. (39). These workers used pulverized magnetic ferric oxide to adsorb influenza viruses from the chorioallantoic fluids of infected embryonated chicken eggs. The adsorbed viruses were eluted by saturated phosphate or carbonate solutions with good yields of viral antigen and substantial reduction of extraneous nitrogenous material. Successful application of this rather simple batch-type procedure to purification of certain myxoviruses suggested its use with HCV and originated the present study.

The absence of such concentration-purification methods for HCV was another limiting factor which frustrated the first attempts to observe HCV particles with the electron microscope. Boynton et al. and Reagan et al. used differential centrifugation to concentrate material containing HCV prior to electron microscopy (6, 7). Although both groups published electron micrographs of particles which may be HCV, Bachrach's observation of similar virus-like particles in extracts of normal pig spleen generates at least some doubt concerning the nature of the virus-like particles described by the other workers (5). In addition to two cycles of differential ultracentrifugation, Bachrach used a methanol precipitation step with infectious spleen extracts, but failed to achieve a state of

purity or concentration sufficient for conclusive electron-micrographic evidence of HCV particles (5). Ageev evidently used thin-sections of embedded erythrocytes from pigs infected with HCV and observed virus-like particles adsorbed to erythrocyte membranes (40). Most recently Mayr and Mahnel obtained good electron micrographs of virus particles from cell cultures infected with cytopathogenic strains of HCV (26). These workers used differential centrifugation to purify HCV prior to concentration in the ultracentrifuge. Unfortunately, their cell cultures were inadvertently contaminated with adenoviruses. Consequently, the electron micrographs show numbers of characteristic adenovirus particles as well as smaller, less well defined picornavirus-like particles about 22 mμ in diameter.

It is evident that our present state of knowledge regarding the physical-chemical properties of HCV is not sufficient for an acceptable classification of this agent. It is also evident that research toward characterization of HCV has been handicapped by the inability to obtain practical quantities of relatively pure and sufficiently concentrated virus preparations.

MATERIALS AND METHODS

Hog Cholera Virus

The virus used throughout this study was an avirulent, noncytopathogenic strain of cell cultured HCV. The original stock culture of this virus was the virulent, cytopathogenic strain A described by Gillespie et al. (22), which was adapted to primary embryonic pig kidney cell cultures by Bass and Ray (24). The virus lost its virulence for swine and its cytopathogenicity after about 100 serial passages in embryonic pig kidney cells (24). The virus is marketed as "Monovac",[1] an avirulent living virus vaccine which successfully immunizes swine against hog cholera (24). "Monovac" virus used in this study originated from the commercial vaccine and was successfully adapted to an established pig kidney cell line. For this study the virus was propagated in cultured cells grown in large flasks, collected aseptically in the fluid culture media, and stored without further treatment at -20°C until needed.

Cell Cultures

The NADL PK-15 (PK-15) swine kidney cell line as described by Pirtle (41), was used to propagate HCV, and also as an

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[1]Monovac; Affiliated Laboratories Corporation, White Hall, Ill.

indicator host for the in vitro titration of HCV. PK-15 cells were maintained by serial passage in screw-capped 250 ml sterile plastic culture flasks[1].

The fluid culture medium used for cell growth and virus propagation was Eagle's minimum essential medium with Earle's balance salt solution and other supplements (MEMS). The formula for MEMS is given as follows:

Component	Grams/L
NaCl	6.800
KCl	.400
$NaH_2PO_4.2H_2O$	.150
$MgSO_4.7H_2O$	.200
$CaCl_2$ (anhyd.)	.200
Glucose	1.000
L-arginine HCl	.126
L-cystine	.024
L-tyrosine	.036
L-histidine $HCl.H_2O$	.042
L-glutamine	.292

[1]Obtained from Falcon Plastics, a division of B-D Laboratory, Inc., 5500 W. 83rd Street, Los Angeles 45, Calif.

Component	Grams/L (continued)
L-isoleucine	.0525
L-leucine	.0524
L-lysine HCl	.073
L-methionine	.015
L-phenylalanine	.033
L-threonine	.048
L-tryptophan	.010
L-valine	.047
Choline Cl	.001
Folic acid	.001
i-inositol	.002
Nicotinamide	.001
Ca-D-Pantothenate	.001
Pyridoxal HCl	.001
Riboflavin	.0001
Thiamine HCl	.001
Phenol red	.010
$NaHCO_3$	2.200

Supplements[1]: L-glutamine, lactalbumin hydrolysate (0.5%), sodium pyruvate (1m M), calf serum (2-10%)[2], penicillin (100 units/ml) and streptomycin (100 μg/ml).

Using MEMS with 2% calf serum, PK-15 cells were maintained in a healthy condition for at least 10 days when refreshed by medium changes at 2-3 day intervals. PK-15 cells used for seeding new cultures were dispersed by replacing the MEMS medium with a trypsin-versene solution (ATV) previously heated to 37°C. The ATV was allowed to react with the cells for about 5 minutes during which time the culture flasks were vigorously shaken at least twice to hasten detachment and to disaggregate cell clumps into single cells. Dispersed cells were poured from the culture flask into conical centrifuge tubes and packed by low speed (1000 rpm) centrifugation at room temperature. After the supernatant ATV was discarded, the packed PK-15 cells were resuspended by gentle pipetting into fresh, pre-warmed MEMS containing 2% calf serum. Twenty-five ml of the fresh medium containing the dispersed PK-15

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[1]Eagle's minimum essential medium plus nonessential amino acids and L-glutamine were purchased as a pre-mixed powder (MEM dry powder medium, F-15) from the Grand Island Biological Co., Grand Island, New York. The complete medium for PK-15 cells was originally suggested by Dr. E. C. Pirtle of the National Animal Disease Laboratory, Ames, Iowa.

[2]Calf serums were obtained from specific pathogen free calves which were colostrum deprived and raised under conditions which exclude all but a minimal chance of exposure to bovine pathogens. Such serums were heat inactivated and known to be free of antibodies against BVD and HCV.

cells were added to 250 ml screw-capped plastic culture flasks and incubated at 37°C. When a single heavy culture was thus dispersed into 5 new cultures, fresh monolayered cultures were established in about 48 hours.

For propagation of HCV, PK-15 cells were cultured in one liter, rubber stoppered, pyrex glass culture flasks. Cell monolayers were established in the one-liter flasks about 48 hours after being seeded with dispersed cells in 100 ml of MEMS containing 2% calf serum. One-liter monolayer cell cultures were infected with $1x10^7$ infectious units of HCV in a 10 ml inoculum pipetted onto each cell monolayer after the growth medium was discarded. Virus in the inoculum was allowed to adsorb to the cells for one hour during which the inoculum was redistributed over the monolayer at 15-minute intervals. At the end of the adsorption period the inoculum was replaced with 100 ml of pre-warmed MEMS containing 10% calf serum. Cultures thus infected were incubated an additional 48 hours at 37°C. At the end of this incubation period the virus-containing fluid medium from several bottles was pooled, divided into aliquots and stored at -20°C until needed. Samples of each virus pool were cultured in NIH thioglycollate broth[1] and Sabouraud's dextrose broth[1] for bacterial and mycotic contaminants. When found such agents were removed

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[1]Dehydrated medium obtained from Difco Laboratories, Detroit 1, Mich.

from the virus pool by filtration through a 0.33 μ Millipore[1] membrane.

When used as indicator hosts for the in vitro titration of HCV, fresh PK-15 monolayer cells were dispersed with ATV and suspended in MEMS containing 2% calf serum. The concentration of suspended PK-15 cells was adjusted so that when 1.5 ml of cell suspension was used to seed each Leighton cell-culture tube, a monolayer cell sheet was established after 24 hours of incubation at 37°C. Each 15 x 125 mm Leighton tube contained a 10.5 x 35 mm glass coverslip for attachment of the cell monolayer, and was sealed with a siliconized latex stopper.

Buffers and Solutions

All buffers and solutions were prepared using volumetric glassware and a precision balance. Except where indicated, buffers and solutions were sterilized by filtration through 0.33 Millipore[1] filter membranes. Samples of buffers and solutions were cultured, when appropriate, in NIH thioglycollate broth[2] and Sabouraud's dextrose broth to confirm freedom from bacterial and mycotic contaminants.

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[1]Millipore Filter Corporation, Bedord, Mass.

[2]Sigma Chemical Company, 3500 DeKalb St., St. Louis 18, Mo., Sigma Technical Bulletin 106B (7-61).

Phosphate-buffered saline (PBS)

Phosphate buffered saline was prepared according to Dulbecco's formula for PBS, listed by Merchant et al. (42):

	Material	Amount	Preparation
Unit #1	NaCl	8.0 Gm	Components of Unit #1 were dissolved in 800 ml of distilled water
	KCl	0.2 Gm	
	Na_2HPO_4	1.15 Gm	
	KH_2PO_4	0.2 Gm	
Unit #2	$CaCl_2$	0.1 Gm	Dissolve in 100 ml distilled water.
Unit #3	$MgCl_2 \cdot 6H_2O$	0.1 Gm	Dissolve in 100 ml distilled water.

Units #1, 2, and 3 were autoclaved separately and mixed after being cooled.

Tris-buffered saline (TBS)

The following formula for 0.05 M Tris buffer, pH 7.4 was taken from a table provided by Sigma[1]:

	Grams/Liter
Trizma HCl	6.61
Trizma base	0.97

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[1]Sigma Chemical Company, 3500 DeKalb St., St. Louis 18, Mo. Sigma Technical Bulletin 106B (7-61).

The 0.05 M Tris buffer, pH 7.4 was diluted 1:5 with distilled water to the 0.01 M level used most frequently in this study. The 0.01 M Tris buffer was used as diluent to prepare 0.146 M NaCl solutions which are designated as 0.01 M TBS.

Physiologic saline solution (PSS)

Physiologic saline was prepared as a 0.146 M solution of sodium chloride in distilled water and was considered physiologic or isotonic with respect to mammalian cells.

Saturated ammonium sulfate

Ninety Gm of ammonium sulfate were added to 100 ml of distilled water, mixed and allowed to stand for 24 hours. The saturated supernatant fluid was used to precipitate gamma globulins from swine serums.

0.1 M glycine buffer, pH 9.7

Glycine buffer in saline was prepared as follows: 7.505 Gm glycine and 5.85 Gm NaCl were dissolved in about 500 ml distilled water. Sodium hydroxide (0.1 N) was added until the pH of the glycine-saline solution had reached 9.7. The volume was then brought to exactly 1000 ml with distilled water. The pH of the 0.1 M glycine buffer was 9.7.

Carbonate-bicarbonate buffer, pH 9.0

Solution A: Na_2CO_3; 5.3 Gm were dissolved in 100 ml of distilled water.

Solution B: $NaHCO_3$; 4.2 Gm were dissolved in 100 ml of distilled water.

Addition of 4.4 ml of Solution A to 100 ml of Solution B gave a pH of 9.0.

Versene-trypsin cell dispersion medium (ATV)

The cell dispersion medium used in this study was essentially the same as the saline-ATV medium described by Madin and Darby, except that antibiotics were deleted and the formula was modified to yield an isotonic solution (43). The modified formula is as follows:

NaCl	8.0 Gm
KCl	0.4 Gm
Dextrose	1.0 Gm
$NaHCO_3$	0.58 Gm
Trypsin	0.5 Gm
Versene	0.2 Gm

These ingredients were dissolved in sufficient distilled water to give a final volume of 1000 ml. Four-tenths of an ml of 5% phenol red in water were added to each liter of ATV.

Antiserums

The anti-hog cholera serum used in the culture medium of all in vitro infectivity assays was kindly supplied by Dr. Wm. L. Mengeling of the National Animal Disease Laboratory at

Ames, Iowa. This antiserum, designated #8194, was prepared in a specific pathogen free (SPF) pig[1] which had been hyperimmunized with both cell culture attenuated and fully virulent strains of HCV (32). The antiserum was heated at 56°C for 30 minutes and stored at -20°C until needed. A number of 1:10 stock dilutions in PBS prepared for routine work were also stored at -20°C when not in use.

The original untreated stock of fluorescein-labelled hog cholera antibody was kindly provided by Dr. E. A. Carbrey of the National Animal Disease Laboratory at Ames, Iowa. This antiserum, designated #7802 was prepared by immunizing SPF pigs with an avirulent HCV and by subsequent hyperimmunization with a fully virulent strain of HCV[2]. Fractionation of hog cholera antiserum and conjugation of its isolated gamma globulin with fluorescein isothiocyanate, has been described by Mengeling et al. (33). This procedure was applied to antiserum #7802 as follows:

Twenty-five ml of antiserum #7802 were placed in a round-bottomed centrifuge tube containing a small plastic-coated magnetic stirring bar. The tube and its contents were vertically fixed in a large beaker containing chipped ice and tap water. The beaker was placed on a magnetic stirring

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[1]Hysterectomy derived, colostrum deprived swine raised under conditions which exclude all but a minimal chance of exposure to swine pathogens.

[2]Carbrey, E. A., Preparation of Hog Cholera Antiserum, Ames, Iowa, National Animal Disease Laboratory. Private Communication. 1967.

apparatus so that the open end of the centrifuge tube was free to receive the tip of a 50 ml burette tube containing a saturated solution of ammonium sulfate. Magnetic stirring assured rapid mixing of the ammonium sulfate and serum while the ice water bath maintained a temperature of 2-4°C within the tube. Saturated ammonium sulfate was allowed to drip slowly into the serum until 12.5 ml had been added. At this point, a fine white precipitate had been produced.

The precipitate was sedimented by centrifugation at 2,500 rpm for 15 minutes at 4°C. Supernatant fluid was discarded and the precipitate redissolved in 25 ml PSS at 4°C. Gamma globulin from antiserum #7802 was thus precipitated a total of 3 times. The final precipitate was redissolved in 12.5 ml of PSS and dialysed overnight at 4°C against 500 ml of PSS. Four changes of fresh PSS were used during the dialysis procedure.

Protein concentration of the dialysed gamma globulin was determined using the Biuret method and diluted with PBS to 10 mg protein/ml of solution. A weighed quantity of fluorescein isothiocyanate (FITC) (isomer B)[1] was dissolved in 6 ml of pH 9.0 carbonate-bicarbonate buffer which, when added to the globulin solution, gave a final concentration of 0.025 mg FITC per mg of protein. The mixture of FITC and globulin

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[1]Baltimore Biological Laboratory, Baltimore 18, Md.

solution was stirred overnight at 4°C.

A 2 x 12 cm cylindrical column of Sephadex G-25[1] was prepared by pouring a slurry of the hydrated gel into a vertically mounted glass chromatography tube. A stock of hydrated Sephadex G-25 was prepared by mixing 100 Gm of the gel with 650 ml PBS. PBS was passed through the gel in the column until pH equilibrium between the buffer and gel was indicated. The solution of FITC-conjugated gamma globulin was freed of unbound FITC by filtration through the gel column. Thus, the excluded conjugate rapidly drained out of the column while unbound FITC was retarded by diffusion into the hydrated gel.

Gamma globulin which non-specifically binds to cell proteins was removed from the conjugate by adsorption with rehydrated rabbit liver powder[2] at the rate of 20 mg per mg of protein in the conjugate. Two and a half ml of PBS was used to rehydrate each gram of desiccated liver powder. The slurry of liver powder and conjugate were allowed to interact by stirring at 4°C for 18 hours. Coarse liver powder material was removed by one cycle of centrifugation at 3,000 rpm for 30 minutes at 4°C. The turbid supernatant conjugate was further clarified by centrifugation at 30,000 rpm for 60

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[1]AB Pharmacia, Uppsala, Sweden.

[2]Generously provided by Dr. Wm. L. Mengeling of the National Animal Disease Laboratory, Ames, Iowa.

minutes in a Spinco Model L preparative ultracentrifuge using the #30 angular rotor. Final clarification was achieved by passing through a 0.33 μ Millipore filter membrane. The conjugate thus prepared was divided into 1 ml aliquots and stored at -20°C until used. For optimum effect, the stock conjugate was diluted 1:5 with 0.01M TBS before use.

Fluorescent-Antibody Plaque Assay (FAPA) Procedure

The FAPA procedure used to quantitate HCV infectivity throughout this study was the same as a procedure originally described by Mengeling (32). PK-15 cultures used in the FAPA procedure, were grown on coverslips in Leighton cell-culture tubes as previously indicated. Just prior to dosing such cultures with the virus dilutions prepared for testing, the fluid medium in each tube was discarded.

For infectivity assays virus samples were prepared as 10-fold serial dilutions in MEMS containing 1% calf serum. A set of two Leighton tube cultures of PK-15 cells was used to assay each dilution of HCV. Each tube received a 0.2 ml dose of the appropriate dilution. The tubes were stoppered, positioned horizontally in Leighton tube racks, and incubated 1 hour at 37°C to permit adsorption of HCV to cells of the monolayer. The fluid inoculum was redistributed over the monolayers at 10-minute intervals during the adsorption period. After the adsorption period the fluid inoculum was decanted

from each tube and replaced with 2 ml of pre-warmed MEMS containing 10% calf serum and 0.1% antiserum #8194. As indicated by Mengeling (32), the 0.1% level of antiserum #8194 was sufficient to prevent all but cell-to-cell dissemination of HCV from primary foci in the cell monolayers. Thus, on a given monolayer, discrete plaques of infected cells developed for each successfully adsorbed infective unit of HCV.

After addition of fresh MEMS containing calf serum and HCV antibody, such tests were incubated 40-48 hours at 37°C. The coverslip monolayers were then removed from the Leighton tubes, rinsed with PBS, and fixed in acetone containing 10% methanol for 10 minutes at 22-24°C. After fixation, the coverslips were placed horizontally across parallel plastic tubes stretched longitudinally over a narrow rectangular wooden pallet. The plastic tubes were smeared with a thin coat of vacuum grease to prevent movement of the coverslips during subsequent treatments. After evaporation of the acetone-methanol fixative, the coverslips were flooded with a thin layer of FITC-conjugated antiserum #7802 and incubated 15 minutes at 37°C in a humid atmosphere. After this incubation period, the coverslips were placed in labelled test tubes containing PBS and allowed to stand at 22-24°C for 5 minutes. Coverslips were then rinsed in distilled water, returned to the coverslip pallet and allowed to dry. In the latter part of this study, it was observed that a superior condition of

the PK-15 monolayers was maintained when the staining procedure was modified as follows: PBS was substituted with 0.01M TBS containing 0.0005M $MgCl_2$ and 0.00015M $CaCl_2$. The same concentrations of these cations was added to distilled water used for rinsing. Coverslip cultures thus prepared were mounted, cell surface downward, on a 5 x 7.5 cm glass microscope slide. The mounting medium used was prepared by mixing equal volumes of glycerol and 0.02M TBS and the above mentioned cations. The upper surface of the glass microscope slide was etched with longitudinal parallel lines 1.5 mm apart[1]. Such lines facilitated a systematic coverage of the superimposed coverslip cultures while enumerating cell plaques stained with fluorescent antibody.

The microscopic enumeration of cell plaques stained with fluorescent antibody was performed with a Leitz Ortholux fluorescence microscope using a BG-12 exciting filter and an OG1 yellow barrier filter. The ultra-violet light source was an HBO-200 W lamp. Use of the darkfield condenser illuminated a darkfield area suitable for low power observation of the coverslip cultures. Infected cell plaques were easily observed with the method described and counts were usually

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[1]Prepared by Dr. Mengeling of the National Animal Disease Laboratory at Ames, Iowa.

[2]E. Leitz, 468 Park Ave. South, New York, New York.

taken from coverslip cultures containing 50-300 plaques. Thus, the number of plaques occurring on such cultures were accumulated on a hand tally while the coverslip was rapidly scanned. A simple average was calculated from counts obtained from the two best coverslips from a dilution series in a given assay. After a correction was made for the dose volume the infectivity titer was expressed as a common logarithm of the reciprocal dilution at which the enumeration was made. The average number of plaques found at such a dilution was included as part of the logarithmic expression. It was often more convenient and meaningful to express such infectivity titers as the scientific notation of their common logarithm. Since each plaque represents a single infective unit of HCV, the infectivity titers in this study are expressed in terms of plaque-forming units (PFU).

Micro-Kjeldahl and Biuret Methods for Organic Nitrogen Determinations

The micro-Kjeldahl method for organic nitrogen was used to determine near-absolute values to establish the degree of purity obtained with the procedure used in this study. The procedure used was taken from a description by Kabat (44) and is as follows:

Reagents

Sulfuric acid 40 ml of a saturated aqueous Cu SO_4 solution was added to a 9 lb bottle of "low N" C.P. reagent sulfuric acid in 10 ml portions with thorough mixing. After several days, the excess anhydrous Cu SO_4 settled out as crystals and the supernatant acid was ready for use.

Potassium sulfate Analytical Reagent Grade, low nitrogen K_2 SO_4 was used.

Boric acid-indicator mixture

Stock solution A 1% methylene blue in water.

Stock solution B A saturated solution of twice recrystallized methyl red in 95% ethanol.

Stock solution C 15 ml of stock solution A was mixed with 125 ml of solution B.

Stock solution D 0.1 ml of solution C was added to 40 ml of saturated boric acid in distilled water.

50% NaOH solution 50% NaOH solution for Kjeldahl nitrogen determinations was obtained from Fisher Scientific Company, Fair Lawn, New Jersey.

Standard N/70 HCl The N/70 solution was made by dilution of exactly N/1.000 HCl. The normality of N/1.000 HCl was determined by direct weighing of constant boiling HCl.

Procedure

Ten ml Pyrex micro-Kjeldahl flasks were used for the digestion of samples. One ml volumes of suitably diluted

samples were added to duplicate sets of digestion flasks. Two or three Hengar granules[1] were added to each flask with about 0.25 Gm of $K_2 SO_4$ and 0.5 ml of $H_2 SO_4$ concentrate saturated with $Cu SO_4$.

The flasks were then placed on a micro-Kjeldahl electric digestion rack and the heat under each flask was cautiously increased until the samples boiled without superheating. Alternatively, samples were pre-heated at 125°C in an oven for 16-18 hours prior to digestion. The alternative step greatly reduced the foaming of samples during the early part of digestion. Samples were allowed to digest under full heat for 30 minutes after acid-reflux rings appeared in the necks of the flasks.

At the end of the digestion period the samples were allowed to cool and 2 ml of distilled water were added to each flask. The crystalline material in each flask was dissolved by gently heating the mixture to 100°C.

The fluid content of each flask was quantitatively transferred to a micro-Kjeldahl glass distillation apparatus (44), and 3 ml of 50% NaOH was quantitatively added to the sample within the apparatus. The mixture of sample and NaOH was then steam distilled for a total of 3 minutes after condensate began to emerge from the condensing unit. Each condensate was

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[1]Boiling chips obtained from the Hengar Company, Philadelphia, Pa.

collected in a tube containing 2.0 ml of Solution D of the boric acid-indicator mixture.

The condensates derived from each sample were titrated with N/70 HCl to a colorless endpoint matching that obtained in a distilled water blank run. Such titrations were carried out using a microburette to deliver the N/70 HCl. Constant stirring was effected by bubbling a stream of nitrogen through the sample as the N/70 HCl was added. Appropriate duplicate sets of control samples were included with each titration. Thus, the organic nitrogen content of a sample could be corrected by subtracting the nitrogen contributed by the sample diluent.

Use of exactly N/70 HCl made nitrogen calculations extremely simple since mg N = ml N/70 HCl x 0.2 x the reciprocal dilution factor of the sample. Therefore, the nitrogen content of a sample was expressed as mg N/ml after a simple average was calculated from the duplicate set of titration values determined for each sample.

Biuret Method

The biuret method for protein nitrogen determinations used in this study has been described by Gornall *et al.* (45). It was used primarily to obtain rapid estimates of nitrogen content in various samples. Such information was especially helpful for determining the proper dilution of samples for

micro-Kjeldahl nitrogen assays. The biuret reagent and the procedure used were as follows:

Reagent

1. 1.5 Gm of finely powdered Cu SO_4 was dissolved in 400 ml of distilled water.

2. 6.0 Gm of sodium potassium tartrate was then dissolved in the 400 ml of Cu SO_4 solution.

3. 300 ml of 2.5 N sodium hydroxide was added to the Cu SO_4-tartrate solution through a 50 ml burette. The NaOH was added dropwise with constant stirring.

4. 1.0 Gm of potassium iodide was dissolved in the mixture.

5. The volume was brought to exactly 1000 ml with distilled water.

Procedure

One ml of suitably diluted sample was mixed with 4 ml of biuret reagent and allowed to react for 30 minutes. The optical density of the reacted mixture was determined with a Bausch and Lomb Spectronic-20[1] spectrophotometer at a wavelength of 540 mμ. Appropriate controls were included with each determination. The protein content of an unknown sample was estimated by consulting a standard plot of optical density

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[1]Bausch & Lomb Incorporated, Rochester, N. Y.

x mg protein obtained from known quantities of a purified protein.

Preparation of Magnetic Ferric Oxide

The magnetic ferric oxide (MFO) used in this study was obtained from the Minerals, Pigments and Metals Division of Charles Pfizer & Co., Incorporated[1]. Three 100 Gm batches of MFO were prepared as follows:

One hundred Gm of MFO were placed in a ball mill and suspended in 1000 ml of distilled water. The ball mill was filled to about one-half its volume with nearly round, smooth stones approximately 0.5 inches in diameter. The ball mill was sealed and operated at 74 rpm for a total of 24 hours. This was sufficient to pulverize the MFO into particles about 1 μ in diameter. The pulverized MFO was transferred to a 5-liter glass-stoppered reagent bottle and washed in about 4 liters of distilled water. After the MFO settled out, the supernatant water was removed by aspiration and replaced with distilled water. The MFO was resuspended in the water and again allowed to settle out. Three batches of MFO were thus washed by 8 such cycles of resuspension and sedimentation in distilled water.

After the final wash cycle, nearly all the supernatant water over the MFO sediment was removed by aspiration and the

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[1]Metal Oxide-9853. Chas. Pfizer & Co., Inc., New York, N.Y. 10017.

thick MFO slurry was transferred to a 1000 ml graduated glass cylinder. The slurry was vigorously shaken to assure uniform distribution of MFO particles and exactly 10 ml were withdrawn and placed in a pyrex weighing dish for dry weight determination. The sample was evaporated to dryness at 100°C for 20 hours. After the dry weight of the sample was determined the slurry was diluted with distilled water to 100 mg MFO/ml of stock suspension. One hundred ml aliquots were transferred to pyrex screw-capped bottles and sterilized in an autoclave.

Certain of the 100 ml aliquots of stock MFO were given an additional treatment by washing twice in a volume of 0.001 M NaCN equal to twice the volume of packed MFO. These aliquots were then washed in distilled water as previously described, diluted to 100 mg MFO/ml and sterilized in an autoclave. Application of cyanide-pretreated MFO to studies with HCV will be described in another section of this report.

Ability of Magnetic Ferric Oxide to Adsorb and Elute Hog Cholera Virus

In the original experiments to determine whether MFO would adsorb HCV, three 5-ml samples of HCV were mixed with 50, 100, and 200 mg MFO, respectively. The mixtures were then agitated on a rotary shaker[1] for 20 minutes at 22-24°C.

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[1]Gyrotory Shaker, New Brunswick Scientific Co., New Brunswick, N.J.

After agitation the MFO was sedimented by centrifugation at 1,500 rpm for 2 minutes and the supernatant fluids were assayed for HCV infectivity. An untreated aliquot of the HCV used in adsorption trials was retained throughout the procedure and assayed as a virus control. As a result of the above preliminary adsorption trials, 40-50 mg MFO/ml of virus were used in all subsequent experiments.

The following solutions were prepared to study their ability to elute HCV adsorbed to MFO:

Na_2HPO_4	5.3%
$NaHCO_3$	10.0%
EDTA[1]	0.02M
NaCN	0.01M

All solutions were made in distilled water except EDTA which was dissolved in 0.01M tris buffer, pH 8.6.

Prior to the elution experiment, the stability of HCV in the phosphate, carbonate, EDTA and the 0.01M NaCN solutions was determined by dialyzing HCV samples against each solution. An aliquot of HCV was divided into 5 samples and each sample was placed in a sac prepared from dialysis tubing[2]. One virus sample was placed in each of 4 flasks containing 100 ml of the eluting solution and one sample was placed in a flask

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[1]EDTA: Disodium Ethylenediamine-tetra acetate.

[2]Union Carbide Corporation, Food Products Division, 6733 W. 65th St., Chicago, Illinois, 60638.

containing PBS as a control. All five samples were then placed on a rotary shaker and dialyzed at 4°C for 24 hours. At 24 hours the sacs were opened and the pH of each virus sample was determined. The sacs were re-sealed and dialyzed at 4°C against several changes of PBS for an additional 24 hours. Finally, the HCV infectivity titer of each sample was determined.

To determine the ability of these eluting mediums to dissociate MFO-HCV complexes, 40 ml of HCV were adsorbed to 1,600 mg of MFO. After the MFO-supernatant fluid was removed by centrifugation, the packed MFO was washed by resuspension in 40 ml of PSS. The MFO was again packed by centrifugation and the supernatant PSS was decanted. This washing cycle was repeated four times and the PSS washings from each cycle were collected as a single pool. During the fourth washing, the MFO slurry was divided into four 10-ml aliquots and centrifuged separately. Each of the resulting MFO pellets was then resuspended in 10 ml of one of the eluting mediums and agitated for 10 minutes on a rotary shaker. The samples were again centrifuged and the supernatant fluids were dialyzed overnight against 400 ml PBS at 4°C. The PBS was changed three times during dialysis procedure. Samples were stored at -20°C until used. Finally, the dialyzed MFO-supernatant fluid, pooled washings, the 4 eluates and a virus control dialyzed against PBS were assayed for HCV infectivity. Results of the elution trial indicated that only the cyanide solution

was effective in dissociating significant quantities of infective HCV from MFO-HCV complexes. Consequently, additional experiments were conducted to study the nature of the interaction between MFO-HCV complexes and cyanide ions.

Effect of Cyanide Concentration on Dissociation of MFO-HCV Complexes

To determine an optimal concentration of cyanide ions for dissociation of MFO-HCV complexes the following molarities of NaCN were prepared in Glycine buffer; pH 9.7: 0.001, 0.005, 0.010, and 0.020.

Fifty ml of HCV was adsorbed to 2,000 mg MFO, centrifuged, and washed with 4 cycles of resuspension and centrifugal sedimentation in 50 ml of cold PSS. The final washing was divided into five 10-ml aliquots which were centrifuged separately. Four of the resulting MFO pellets were resuspended in 10 ml of one of the cyanide-glycine buffer solutions while the fifth pellet was resuspended in 10 ml of glycine buffer alone. The five slurries were agitated for 10 minutes and centrifuged to pellet the MFO. Each supernatant eluate and a virus control were dialyzed overnight at $4^{o}C$ against 4 separate 400 ml quantities of cold PBS. After dialysis, the MFO-supernatant fluid, pooled washings, each eluate and the virus control were assayed for HCV infectivity.

Effect of pH on Dissociation of MFO-HCV Complexes With Cyanide Ions

In this experiment 50 ml of HCV were adsorbed to 2,000 mg of MFO. Using methods described in the previous experiment, the MFO was packed, separated from its supernatant fluid phase and washed 4 times in PSS. The fourth washing was divided into five 10 ml portions and each of the resulting MFO pellets were resuspended in 10 ml of one of the following solutions:

NaCN 0.01M in 0.01M Tris-buffer, pH 7.2
NaCN 0.01M in 0.01M Tris-buffer, pH 7.6
NaCN 0.01M in 0.01M Tris-buffer, pH 8.0
NaCN 0.01M in 0.01M Tris-buffer, pH 8.4
NaCN 0.01M in distilled water, pH 9.7

The 5 MFO slurries were agitated for 10 minutes on a rotary shaker and then centrifuged to pellet the MFO. The supernatant eluates were then dialyzed overnight against several changes of PBS at $4^{\circ}C$. A dialyzed virus control and samples from each part of the experiment were assayed for HCV infectivity.

Effect of Sodium Chloride Concentration on Dissociation of MFO-HCV Complexes with Cyanide Ions

The methods used in this experiment were identical to those described for the preceding test. Thus, 50 ml of HCV

were adsorbed to 2,000 mg MFO and subsequently washed in PSS. Three of the 5 equal MFO pellets obtained by division of the final wash slurry were resuspended in 10 ml of 0.05 M, 0.03 M or 0.01 M NaCl in distilled water. One of the two remaining MFO pellets was resuspended in 10 ml of 0.01M NaCN in distilled water, while the other was resuspended in 10 ml of distilled water alone. After agitation for 10 minutes, the slurries were centrifuged and the supernatant eluates dialyzed overnight against PBS as previously described. Dialyzed samples representing the virus control, MFO-supernatant, pooled washings and the 5 eluates were then assayed for HCV infectivity.

Repetitious Elution of MFO-HCV Complexes with Cyanide Ions

A number of experiments were conducted to determine the effect of repetitious elution of MFO-HCV complexes on the total yield of HCV. The methods used for adsorption and washing of MFO-HCV complexes were the same as described in previous experiments. However, a single MFO pellet was produced from the final wash cycle. The MFO pellet was then resuspended in a volume of 0.01M NaCN equal to 1/4 (4 elutions) or 1/3 (3 elutions) the volume of the original virus sample that was adsorbed. The slurry was agitated for 10 minutes then centrifuged to collect the eluate. This procedure was repeated 2 or 3 times depending on the number of eluates desired. The eluates as well as samples representing a virus control, MFO

supernate and pooled washings were dialyzed against PBS and assayed for HCV infectivity as previously described.

Effect of Low Ionic Strength and pH 9.8 on Dissociation of MFO-HCV Complexes

Two replicate experiments were conducted to compare the infectivity yields obtained by dissociating MFO-HCV complexes with 0.01M cyanide (pH 9.8) and with 0.003M NH_4OH in distilled water (pH 9.8). Twenty ml of HCV was adsorbed to 800 mg of MFO and washed in PSS as previously described. The final washing was divided into two 10 ml portions and centrifuged. One of the MFO pellets was eluted twice with 5 ml 0.01M cyanide in distilled water while the other was eluted twice with 5 ml of 0.003M NH_4OH in distilled water. The eluates, virus control, MFO-supernatant and pooled washings were dialyzed against PBS and assayed for HCV infectivity as previously described.

Adsorption and Elution of HCV Using MFO Pretreated with Cyanide Ions

To reduce the amount of colloidal ferric oxide generated by eluting MFO-HCV complexes with 0.01M NaCN, some of the MFO stock was pretreated with 0.001M NaCN as previously described.

The methods used for adsorption of HCV to cyanide-pretreated MFO, the subsequent washing and repetitious elution procedures with 0.01M NaCN were the same as described for

non-pretreated MFO. The method for dialysis against PBS and the assay for HCV infectivity also were described previously.

Attempts to Concentrate HCV by Sequential Re-adsorption and Elution with MFO

Results presented later in this report indicate that 40 mg MFO/ml of virus would adsorb essentially all infectious HCV in a given sample. Also, nitrogen analyses indicate that the virus preparations used in this study contained 1.67 mg N/ml. These data suggested that about 25 mg MFO/ml H in a virus sample were required for efficient adsorption of HCV. Thus, since a single adsorption-elution cycle on MFO removed 90-95% of organic N, it was reasoned that sequential readsorption and elution using MFO at the rate of at least 25 mg/mg N might provide a means of concentration as well as purification of HCV. Consequently, the following experiment was conducted to test the hypothesis concerning the weight ratio of MFO/N as it would apply to a concentration procedure for HCV:

> 10 ml of HCV partially purified on MFO, containing 0.25 mg N/ml, was re-adsorbed to 100 mg of MFO. The MFO-supernate, pooled washings and two eluates were produced as previously described. All samples were dialyzed against PBS and assayed for HCV infectivity. A control experiment was conducted using identical procedures except that the 10 ml of partially purified HCV was re-adsorbed to 400 mg of MFO; a w/w ratio identical to that used in the original purification procedure.

Partial Purification of Large Volumes of HCV Using MFO

A single 100-ml volume and two 300-ml volumes of HCV were adsorbed to cyanide-pretreated MFO and eluted with 0.01M cyanide in distilled water. In each case MFO was added to give a final concentration of 40 mg/ml of virus. The procedure for adsorption and washing in PSS were as described previously. However, the MFO complexes were eluted only twice, and each time with a volume of 0.01M cyanide equal to 1/2 of the volume of virus that was adsorbed. Thus, the combined volume of the two eluates was equal to the original volume of virus adsorbed with MFO. Also, the eluates and other samples were dialyzed against 0.01M TBS instead of the usual PBS. Assays for HCV infectivity with appropriate samples were conducted as previously described.

Density Gradient Centrifugation Experiments

Various concentrations of cesium chloride[1] and sucrose[1] were prepared on a w/w basis in 0.01M TBS for the density gradients used in these experiments. Precise weights from standard tables for cesium chloride (46) and sucrose (47)

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[1]Optical grade cesium chloride was obtained from the Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y. Reagent grade sucrose was obtained from Merck & Co., Inc., Rahway, N. J.

were used to obtain a number of solutions with densities covering the expected range of components in MFO-purified HCV preparations.

Using a precision balance and calibrated volumetric glassware, the density of 0.01M TBS was found to be 1.0023. Therefore, only a minor error was committed by assuming that the standard densities of cesium chloride and sucrose in water alone were equivalent to their densities in 0.01M TBS. Also, standard tables for sucrose (47) indicates a difference of 0.0012 density units for an 8°C change in temperature. Thus, the density variation introduced by conducting density gradient experiments at 10-18°C and determining refractive indices at 21°C, is not considered to be significant. Consequently, density units were recorded using three decimal places.

The following standard densities of cesium chloride and sucrose were prepared in 0.01M TBS:

Cesium chloride	Sucrose
1.088	1.103
1.125	1.127
1.163	1.151
1.217	1.190
1.271	1.229
1.389	1.257
1.508	1.286

The refractive indices of these standards were determined at 21°C with an Abbe Model A Refractometer[1] and plotted as a function of their densities. The plots for both cesium chloride and sucrose indicated a linear relationship between density and refractive index throughout the range given for each substance. Thus, the density for a sample containing either substance was easily determined from its refractive index by consulting the appropriate standard plot. Use of the Abbe refractometer was also advantageous since only two drops of a sample were required to determine its refractive index.

Basically, two types of density gradient centrifugation experiments were used with both cesium chloride and sucrose solutions. A rate-zonal type of experiment was used to concentrate partially purified HCV by sedimentation onto a dense layer of cesium chloride or sucrose solution. In such experiments, 45-50 ml samples of virus were centrifuged onto 6 ml of 28% or 45% cesium chloride or 9 ml of 60% sucrose. When necessary, the volume in the tube containing HCV was adjusted to the volume used in the two balance tubes by addition of 0.01M TBS. The Spinco[2] SW-25.2 rotor was used in the Spinco

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[1]Carl Zeiss, Oberkochen, Wuerttemberg, West Germany.

[2]Spinco Division, Beckman Instruments, Inc. Stanford Industrial Park, Palo Alto, California.

Model L-2 preparative ultracentrifuge operated at 25,000 rpm for 6 hours at 18°C, without use of the braking mechanism. Since the 6-hour duration of centrifugation is sufficient to allow considerable diffusion of a dense medium into areas of much lower density, non-abrupt density gradients were obtained, especially with cesium chloride.

At the conclusion of a run the bottoms of the cellulose nitrate centrifuge tubes were punctured with a 20-gauge needle and the contents were collected as a number of fractions using the needle as a drip-out cannula. Two or three drops of each sample were used to determine the refractive index at 21°C, after which the fractions were dialyzed against several changes of 0.01M TBS at 4°C. After dialysis, the samples were assayed for HCV infectivity, and stored at -20°C until needed.

The buoyant density of partially purified HCV was studied in a second type of density gradient experiment designated, isopycnic density centrifugation. The virus samples used in the isopycnic experiments were the rate-zonal centrifugation fractions with the highest infectivity titers. Such samples were prepared for the isopycnic experiments by adjusting the density to about 1.15 with either 45% cesium chloride or 60% sucrose. Five ml of virus thus prepared were placed in a 5 ml cellulose nitrate tube and centrifuged at 39,000 rpm in the Spinco L-2 preparative ultracentrifuge using the SW-30 L rotor. When cesium chloride was the density medium, isopycnic experiments were centrifuged at 18°C for 24 hours,

whereas, with sucrose the centrifuge was operated at 10°C for 48 hours. Immediately after centrifugation the bottoms of the centrifuge tubes were cannulated with a 22 gauge needle and the contents collected drop-wise in 0.5 ml fractions. After the tubes were emptied, a pellet sample was obtained by resuspending the pelleted material in 0.5 ml of 0.01M TBS. As previously described, the refractive index of all but the pellet sample was determined and the samples were dialyzed against 0.01M TBS at 4°C. Finally, all fractions were assayed for HCV infectivity.

Preparation of HCV for Electron Microscopy

Samples of HCV used in electron microscopic studies were those isopycnic density fractions with the peak infectivity titers and the resuspended pellet material. Such samples were dialyzed for 18-24 hours at 4°C in distilled water to reduce salt concentrations to a negligible level. After dialysis, 2-3 drops of the virus were mixed with 2 drops of 2% aqueous potassium phosphotungstate (pH 6.7 to 7.2) and 1 drop of about .01% aqueous bovine serum albumin. This mixture was placed in a nebulizer and sprayed onto carbon-coated collodion filmed grids. HCV preparations, thus negatively stained, were immediately examined in a Phillips EM 200 electron

microscope at 60 kv with double condenser illumination[1]. In addition, two-drop aliquots of such HCV samples dialyzed in distilled water were mixed with 1-2 drops of FITC-conjugate #7802 which had been diluted 1:250 in distilled water. After incubation overnight at 4°C the HCV-antiserum mixture was negatively stained and observed in the electron microscope.

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[1]The negative staining procedure and operation of the electron microscope were conducted by A. E. Ritchie at the National Animal Disease Laboratory, Ames, Iowa.

RESULTS

Ability of MFO to Adsorb and Elute Infectious Hog Cholera Virus

The capacity of MFO to adsorb HCV was determined in one or more preliminary trials with each 100 Gm batch of MFO. Results of these trials are summarized in Table 1. The data shown for each batch was determined subsequent to the sixth day after the MFO was sterilized. These data indicate that each MFO batch behaved similarly and that 40 mg MFO/Ml of virus was sufficient to adsorb nearly all the HCV in each sample.

Table 1. Capacity of MFO for adsorbing HCV

	Mg MFO per ml of virus		
MFO sample	10	20	40
Batch #1	0.58[a] (74)[b]	1.93 (98.8)	2.98 (99.9)
Batch #2	0.90 (87)	2.17 (99.33)	2.87 (99.86)
Batch #3	0.48 (67)	1.90 (98.8)	2.73 (99.82)

[a] Log_{10} difference between virus control titer and titer of non-adsorbed virus in MFO supernatants.

[b] Percent of virus adsorbed to MFO.

Table 2. Stability of HCV in eluting solutions

Solutions[a]	pH at 24 hours	Log_{10} titer per ml[c]
Control virus in PBS	7.4	5.47
10% $NaHCO_3$	8.1	5.30
5.3% Na_2HPO_4	8.7	5.23
0.02M EDTA[b]	8.1	5.20
0.01M NaCN	9.7	5.13

[a] 5 ml virus samples dialyzed at 4°C against indicated solutions.

[b] EDTA made up in 0.05M Tris buffer at pH 8.6.

[c] After additional 24 hours dialysis against PBS, pH 7.4.

The results of a preliminary trial to determine the stability of HCV infectivity in selected eluting solutions are shown in Table 2. These data indicate that HCV was relatively stable to a prolonged treatment with the four eluting solutions. Consequently, the capacity of each eluting solution for dissociating MFO-HCV complexes was tested in an experiment summarized in Table 3. The results of this experiment indicate that only the 0.01M NaCN solutions eluted a substantial quantity of infectious HCV. This finding suggested some specific activity of cyanide ions and led to more detailed experiments bearing on the dissociation of MFO-HCV complexes

Table 3. Ability of selected eluting solutions to dissociate MFO-HCV complexes

Sample	Total PFU x 10^5	% PFU yield
Virus control	14.8	100
MFO supernate	< .01	< 0.1
Pooled washings	< .015	< 0.1
(1) 10% $NaHCO_3$	< .01	< 0.1[a]
(2) 5.3% Na_2HPO_4	< .01	< 1[a]
(3) 0.02M EDTA	.015	0.1[a]
(4) 0.01M NaCN	1.36	9.2[a]

[a]Yields were determined from a single elution.

with NaCN.

The first experiment concerning the activity of cyanide ions was designed to determine an optimal concentration of NaCN for dissociating MFO-HCV complexes. Since 0.01M NaCN in water usually had a pH of 9.7-9.8, the various concentrations of NaCN used were prepared in 0.1M glycine buffer, pH 9.7. The result of dissociating MFO-HCV complexes with various concentrations of buffered NaCN is given in Table 4. These data indicate that the 0.01M NaCN was the most efficient eluting solution. However, it should be noted that since glycine buffer did not control pH in the 0.02M NaCN solution,

Table 4. Effect of cyanide concentration on dissociation of MFO-HCV complexes

Sample	pH	Total PFU x 10^5	% yield
Virus control	7.4 (PBS)	150	100
MFO supernate	ND[b]	< 0.02	<0.1
Pooled washings	ND	<0.05	<0.1
Eluates			
0.1M Glycine buffer	9.7	0.25	0.17
0.001M NaCN + GB[a]	9.6	26	17.6
0.005M NaCN + GB	9.7	27.5	18.6
0.010M NaCN + GB	10.0	32.5	23.8
0.02M NaCN + GB	10.3	19.0	12.7

[a]All NaCN solutions made in 0.1M glycine buffer, pH 9.7.

[b]ND = Not done.

the poor yield in this sample may have been due to effects of high pH. Also notable is the fact that glycine buffer alone eluted 0.17% of the total PFU yield while 0.005M NaCN at the same pH, eluted 100-fold more virus. This further suggests a specific eluting activity for cyanide ions and indicates that dissociation of MFO-HCV complexes is only partially dependent on pH.

Table 5. Effect of pH on dissociation of MFO-HCV complexes

Sample	Total PFU x 10^5	% yield
Virus control	47.5	100
MFO supernate	0.10	0.21
Pooled washings	0.52	1.1
Eluates		
NaCN-Tris 7.2[a]	0.09	0.35
NaCN-Tris 7.6	0.11	0.75
NaCN-Tris 8.0	0.75	1.57
NaCN-Tris 8.4	3.53	7.5
NaCN-HOH 9.7[a]	22.0	46

[a]Cyanide (0.01M) made up in 0.01M Tris buffer at pH indicated. pH of 0.01M NaCN in water was 9.7.

In order to determine the effect of pH on the dissociation of MFO-HCV complexes, samples of 0.01M NaCN prepared in 0.01M Tris buffers at various pH values were used as eluting solutions. The results of this experiment are summarized in Table 5. These data show a progressive increase in yield of HCV infectivity as pH is increased. A most striking feature of these data is the large increase in yield between pH 8.4 and 9.7. Since data presented in Table 4 indicate decreasing

HCV yields at pH 10.3, it is evident that a pH of about 9.7-10.0 is optimal for dissociating MFO-HCV complexes with 0.01M NaCN.

Since it was considered undesirable to expose HCV to the osmotic shock produced by the abrupt change from isotonicity in the washing procedure to the hypotonic conditions of the 0.01M NaCN eluting solution, an experiment was conducted to test the effect of NaCl concentration on the MFO-HCV dissociation phenomenon. The results of this experiment, shown in Table 6, indicate that yields of HCV infectivity increased as the saline concentration was decreased. Thus, it was noted that both ionic strength and pH were factors affecting the stability of the MFO-HCV complexes. Indeed, distilled water alone eluted a significant quantity of HCV. Although the pH of the distilled water eluate was not determined, it was observed that supernatant water from MFO washings usually had a pH of 8.4-8.6. Thus, by assuming that a similar pH value applied to the distilled water eluate shown in Table 6, it was reasoned that distilled water when adjusted to pH 9.7 would be far more effective as an eluting solution.

This reasoning led directly to the two experiments which compared the effects of eluting MFO-HCV complexes with 0.01M NaCN and 0.003M NH_4OH in distilled water. The 0.003M concentration of NH_4OH was selected since it gave pH values similar to 0.01M NaCN (9.7-10.0). Results of these experiments are shown in Table 7.

Table 6. Effect of NaCl concentration on dissociating MFO-HCV complexes

Sample	Total PFU x 10^5	% yield
Virus control	225	100
MFO supernate	0.32	0.14
Pooled washings	0.015	<0.1
Eluates[a]		
#1, 0.05M NaCl	5.18	2.3
#2, 0.03M NaCl	22.75	10.1
#3, 0.01M NaCl	48.00	21.3
#4, 0.01M NaCN	107.5	48.0
#5, distilled water	32.5	14.0

[a]Each NaCl concentration contained 0.01M NaCN.

Table 7. Ability of 0.003M NH_4OH to dissociate MFO-HCV complexes

Samples	Total PFU x 10^5		% yield	
	(Exp. A)	(Exp. B)	(Exp. A)	(Exp. B)
Virus control	54	12.8	(100)	(100)
MFO-supernate	.001	<.01	<.01	-
Pooled washings	.004	<.01	<.04	-
NaCN Eluates[a]				
#1	6.8	3.0	12.6	23
#2	18.8	4.0	35.0	31
NH_4OH Eluates[a]				
#1	19.2	4.2	35.6	33
#2	20.4	20	38.0	156[b]

[a]0.01M NaCN had a pH of 9.8 as did 0.003M NH_4OH.

[b]Yield was high but accuracy of this figure is questionable.

These data indicate that use of weak NH_4OH solutions is at least as effective as 0.01M NaCN and has the additional advantage of being generally less toxic and easily neutralized by weak acids.

Effect of Repetitious Elutions on Dissociation of MFO-HCV

Upon recognition that the dissociation of MFO-HCV (complexes) was dependent on low ionic strength, it was reasoned that residual NaCl from the washing procedure might be sufficient to suppress complete release of HCV when only one or two elution steps were used. Therefore, several experiments were conducted to determine the number of elution steps required for maximal yields of infective HCV. The results of three typical experiments are summarized in Table 8.

Generally, these data indicate that two or three elution steps with NaCN will recover most of the available HCV bound to MFO. Low yields consistently obtained with the fourth elution step resulted in its deletion in practical procedures. Yields of 109.4% and 41.4% were included in Table 9 to illustrate the range of yields that were obtained with this procedure. The 109% yield indicates that essentially all infectivity was recovered. Most often total yields were between 50-75%.

Table 8. Effect of repetitious elution steps on dissociation of MFO-HCV complexes

Samples	% yield of PFU		
	(Exp. A)	(Exp. B)	(Exp. C)
Virus control	(100)	(100)	(100)
MFO-supernate	0.1	0.2	<0.1
Pooled washings	<0.1	<0.1	<0.1
NaCN Eluates[a]			
#1	33.5	13.0	5.8
#2	57.0	17.4	48.8
#3	12.2	9.3	8.8
#4	6.7	1.7	2.0
Totals	109.4%	41.4%	65.4%

[a]0.01 M NaCN, pH 9.8.

Effect of Aging on the Adsorption of HCV by MFO

The preliminary experiments concerning adsorption of HCV by MFO were conducted, entirely by coincidence, using material from batch #1 subsequent to the 4th day after sterilization. However, the first adsorption tests with MFO batches #2 and #3 were conducted on the same day or one day after sterilization. In each case MFO batches #2 and #3 failed to adsorb the expected quantities of HCV. When these adsorption

Table 9. Effect of aging on the capacity of MFO to adsorb HCV (x) days after sterilization

	(5)	(19)	(26)	(150)
MFO Batch #1	2.13[a]	2.98	3.9	5.7
	(0)	(12)	(26)	
MFO Batch #2	0.81	2.87	3.98	---
	(1)	(5)	(14)	
MFO Batch #3	0.34	2.73	3.06	---

[a]Log_{10} difference between virus control and titer found in MFO-supernate on day indicated. All values determined with mg MFO/ml of virus.

experiments were repeated a few days later the results indicated an increased avidity of the MFO for HCV. Thus, when the data for each of the three MFO batches were studied and compared with respect to an increased adsorption capacity with aging, it was possible to arrange these data as shown in Table 9.

When compared in this way it is evident that the capacity of MFO for adsorption of HCV increases significantly with time, especially during the first 4-5 days after sterilization. Although the cause of this phenomenon is problematic, recognition of such behavior has obvious importance in any future chromatographic studies with MFO.

Attempt to Concentrate HCV by Sequential Adsorption and Elution with MFO

As indicated in Materials and Methods, a hypothesis concerning the w/w ratio of MFO and mg N in a virus sample was tested to determine if it was possible to concentrate partially purified HCV on MFO with a sequential readsorption-elution procedure. Since partially purified HCV contains only 5-10% of the original organic N, it was reasoned that much less MFO would be needed to readsorb HCV. Thus, through a sequential readsorption-elution series the volume of eluate could be reduced to obtain a concentrated and purified HCV preparation. The results of the controlled experiment designed to test this hypothesis are summarized in Table 10.

The results of this experiment indicate that concentration of HCV is not possible using a sequential readsorption-elution procedure. The data shown for Exp. A indicate that the 100 mg MFO used was not sufficient to adsorb a significant amount of virus while in Exp. B, 400 mg MFO adsorbed essentially all the HCV in the sample. This indicates that the protein and HCV eluted from the original MFO-HCV complex require the same quantity of MFO for complete readsorption. Thus, although HCV can be successfully readsorbed and eluted from MFO, the procedure as described would not be useful for the concentration of HCV.

Table 10. Ability of MFO to concentrate HCV by sequential readsorption and elution

Sample	Total PFU x 10^5		% yield	
	(Exp. A)[a]	(Exp. B)[a]	(Exp. A)	(Exp. B)
Virus control	66	67	(100)	(100)
MFO supernate	65	<0.1	98.4	---
Pooled washings	2.8	.76	4.2	1.1
NaCN eluates				
#1	2.3	49	3.5	73
#2	.98	10.5	1.5	16

[a]In Exp. A, 10 ml of partially purified HCV adsorbed to 100 mg MFO, while in Exp. B (control) such virus was adsorbed to 400 mg MFO which was equivalent to that used in the original purification procedure.

Effect of Pretreating MFO with Cyanide Ions on Dissociation of the MFO-HCV Complex

As previously indicated, the dissociation of MFO-HCV complexes with 0.01M NaCN is accompanied by the apparent generation of colloidal MFO. Thus, the eluates had a distinct dark amber color, the bulk of which precipitated as MFO pellets after the sample had been dialyzed against PBS. Since it was known that MFO would adsorb HCV from isotonic saline solution the presence of colloidal MFO in the eluates was considered undesirable. Consequently, it was found that the pretreatment

of MFO with 0.001M NaCN substantially reduced the amount of colloidal forms appearing in eluates produced with 0.01M NaCN. Thus, two experiments were conducted to study the capacity of cyanide-pretreated MFO for adsorbing and eluting HCV. The results of these experiments are summarized in Table 11.

Table 11. Ability of cyanide-pretreated MFO to adsorb and elute HCV

Samples	Total PFU x 10^5		% yield	
	(Exp. A)	(Exp. B)	(Exp. A)	(Exp. B)
Virus control	0.9	64	(100)	(100)
MFO supernate	<0.01	5	---	7.8
Pooled washings	<0.01	2.9	---	4.5
NaCN eluates[a]				
#1	0.45	71.5	50	111
#2	0.001	0.2	0.1	0.3
#3	<0.001	0.24	---	0.4

[a]Three repetitious elutions in 0.01M NaCN.

Although these data suggest that cyanide-pretreated MFO adsorbs HCV somewhat less avidly than when non-pretreated, it is important to note that the bulk of the HCV infectivity was recovered in the first eluates. This latter feature is in

contrast to results obtained with non-pretreated MFO (Table 8). In view of these findings, subsequent experiments were conducted with cyanide-pretreated MFO using two repetitious elutions in 0.01M NaCN.

Partial Purification of Large Volumes of HCV Using Cyanide-Pretreated MFO

The conditions for adsorbing HCV to cyanide-pretreated MFO and its subsequent elution with 0.01M NaCN have been described in Materials and Methods. Such methods were applied as a partial purification procedure to a single 100 ml and two 300 ml aliquots of HCV in preparation for further purification and concentration by density gradient centrifugation. The results of these three partial purification experiments are summarized in Tables 12-A and 12-B.

Among the salient features of these three experiments is the fact that 40-60% of the original infectivity was obtained with two elutions when volumes common in preliminary experiments were increased about 10-fold. The micro-Kjeldahl nitrogen determinations indicate that 13, 7.5 and 12.5% of the total nitrogen was adsorbed in Exps. #15, 17 and 18, respectively, while 6.3, 6 and 8% of the total nitrogen was eluted in the first eluates. Thus, since the first eluate in each experiment was one-half the volume of original virus, Exp. #15 provided 50 ml of HCV which assayed 4.1×10^6 PFU/ml,

Table 12-A. Partial purification of large volumes of HCV using cyanide-pretreated MFO

Sample	Total PFU x 10^5			% yield		
	(Exp. #15)[a]	(Exp. #17)[a]	(Exp. #18)[a]	(Exp. #15)	(Exp. #17)	(Exp. #18)
Virus control	4,400	9,450	4,500	(100)	(100)	(100)
MFO supernate	6.2	150	16.5	.14	7.9	.37
Pooled washings	33.2	360	126	.75	3.8	2.8
NaCN eluates[b]						
#1	2,050	3,040	2,100	46.6	32.2	47
#2	600	855	555	13.6	9.0	12

[a]Experiment #15 involved 100 ml of HCV while Exps. #17, 18 were 300 ml volumes of virus.

[b]Two repetitious elutions in 0.01M NaCN.

while Exps. #17, 18 provided 150 ml with 2 x 10^6 and 3 x 10^6 PFU/ml, respectively. Since these eluates contained only 6-8% of the original total micro-Kjeldahl nitrogen, such preparations were considered partially purified and suitable for application in physical-chemical studies.

Table 12-B. Micro-Kjeldahl nitrogen determinations for experiments #15, 17, and 18

Sample	Exp. #15	Exp. #17	Exp. #18
Virus control	165.60[a]	481.20	499.2
MFO supernate	127.60	411.60	371.4
Pooled washings	16.48	34.02	64.62
NaCN eluates			
#1	10.49	38.64	40.44
#2	6.49	25.08	29.37

[a]Total mg micro-Kjeldahl nitrogen (volume x mg N/ml).

Density Gradient Centrifugation Experiments

As previously indicated, density values for these experiments were estimated from plots of the refractive indices determined from standard densities of cesium chloride and sucrose in 0.01M TBS. These densities and their corresponding refractive indices are presented in Table 13.

The plots for these values (Figs. 1 and 2) indicate a linear relationship over the range of standard densities used. Thus, densities of unknown samples were obtained simply by consulting the appropriate standard plot. Since the standard plots were linear, it was also possible to calculate the slope

Figure 1. Linear relationship between the refractive indices and standard densities of cesium chloride in 0.01M TBS.

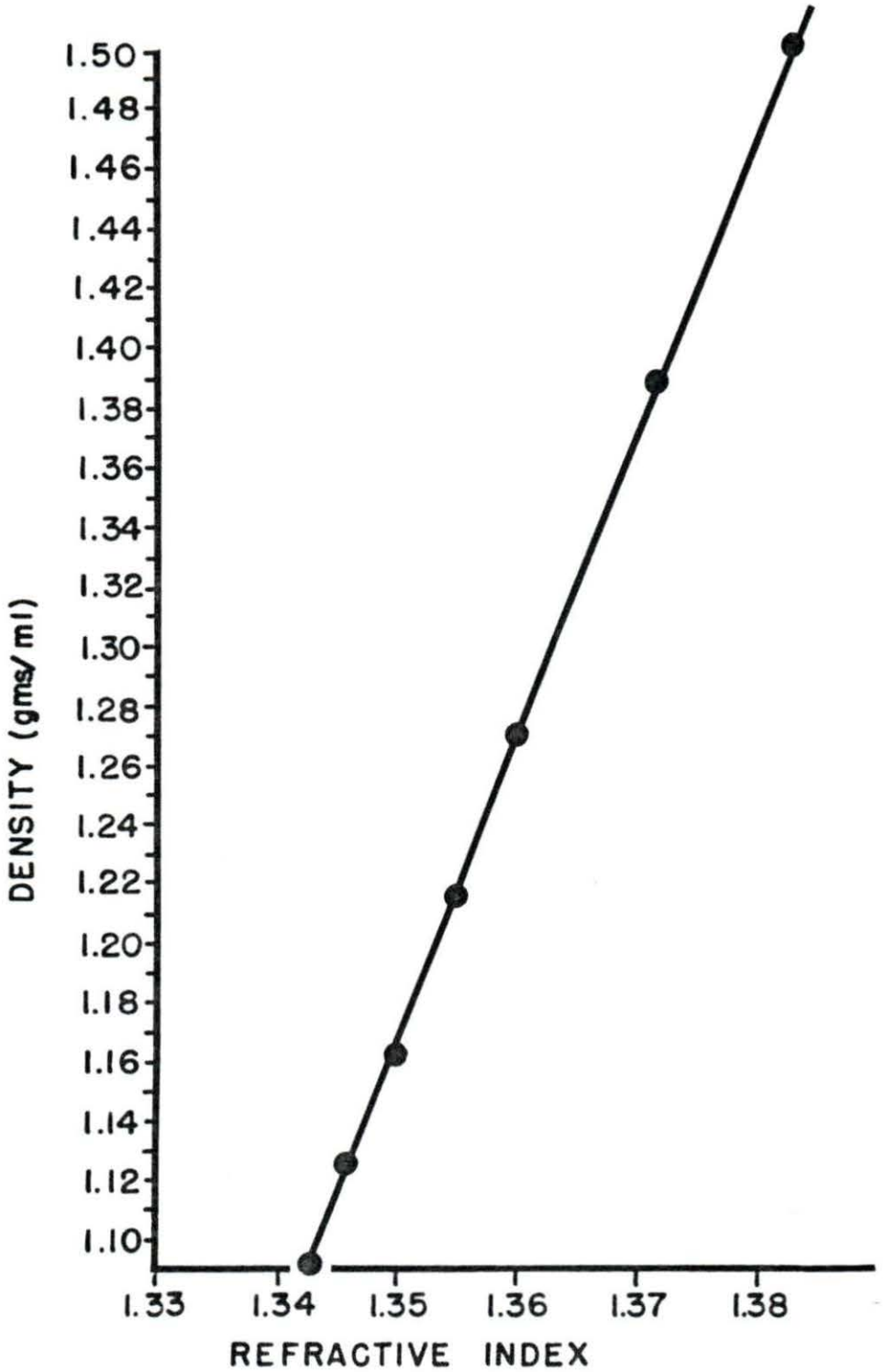

1.50
1.48
1.46
1.44
1.42
1.40
1.38
1.36
1.34
1.32
1.30
1.28
1.26
1.24
1.22
1.20
1.18
1.16
1.14
1.12
1.10
DENSITY (gms/ml)
1.33
1.34
1.35
1.36
1.37
1.38
REFRACTIVE INDEX

Figure 2. Linear relationship between the refractive indices and standard densities of sucrose in 0.01M TBS.

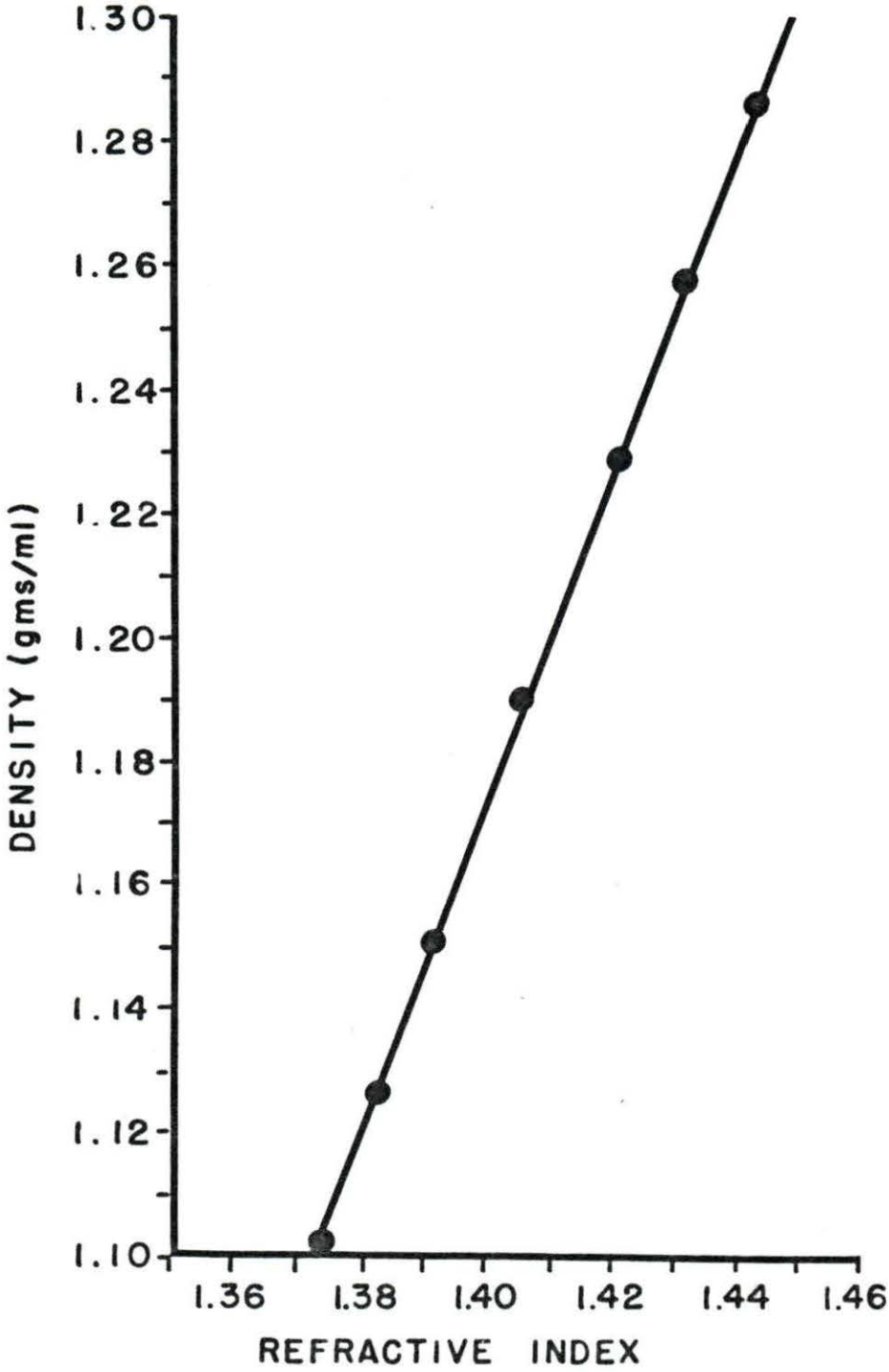

1.30
1.28
1.26
1.24
1.22
1.20
1.18
1.16
1.14
1.12
1.10
DENSITY (gms/ml)
1.36
1.38
1.40
1.42
1.44
1.46
REFRACTIVE INDEX

Table 13. Refractive indices of standard densities for cesium chloride and sucrose in 0.01M TBS

Cesium chloride		Sucrose	
Density[a]	Refractive index[b]	Density	Refractive index
1.088	1.3428	1.103	1.3737
1.125	1.3462	1.127	1.3823
1.163	1.3497	1.151	1.3917
1.217	1.3550	1.190	1.4060
1.271	1.3602	1.229	1.4212
1.389	1.3716	1.258	1.4312
1.508	1.3824	1.286	1.4429

[a]In gm/ml on a w/w basis.

[b]Fourth decimal place estimated with Abbe Model A Refractometer.

and intercept of each using the general formula for a linear equation in two unknowns:

$$y = ax + b,$$

where y = density and x = refractive index

then a = intercept and b = slope.

By inserting data for cesium chloride from Table 13 into the formula and solving for a and b, a = 10.6061 and b = -13.154. Similarly, for sucrose in 0.01M TBS, a = 2.669 and b = -2.5623. As expected, calculated values and values from

the standard plots indicate excellent agreement in the first three decimal places.

Rate-zonal density gradient centrifugation

The first eluates from Experiments 15, 17 and 18 were the source of partially purified HCV used in all density gradient experiments. Four rate-zonal types of experiments were completed, three of which were conducted in cesium chloride, while the fourth was in sucrose. All four rate-zonal experiments were centrifuged at 25,000 rpm for 6 hours at 18° C in the SW-25.2 rotor. The data from these experiments are summarized in Tables 14, 15, 16 and 17.

The results indicate that with both density mediums, HCV was concentrated into relatively narrow bands which were collected as 1-3 ml fractions. Thus, use of the one-step, rate-zonal procedures resulted in a 15-50 fold concentration of HCV. However, recovery of only 15-58% of the total input of PFU (Tables 14-16) in the cesium chloride experiments suggested excessive degradation of HCV by this salt solution. Consequently, a similar run was conducted using 9 ml of 60% sucrose as the initial density medium. Results of this single run (Table 17) indicate that 87% of the input PFU was recovered. These data suggest that sucrose is superior to cesium chloride as a density medium for rate-zonal density gradient concentration of HCV.

Table 14. Density gradient centrifugation of partially purified HCV into cesium chloride[a]

Sample	Fraction vol. (ml)	Total PFU x 10^5[b]	% yield[c]	Density[d]
Virus control	45	1080	(100)	--
Fraction 1	0.5	.13	-	1.18
Fraction 2	0.5	.75	.02	1.17
Fraction 3	0.5	5.5	3.3	1.15
Fraction 4	0.5	65.0	39.4	1.14
Fraction 5	0.5	52.5	31.5	1.13
Fraction 6	0.5	15.0	9.1	-
Fraction 7	0.5	8.0	4.9	-
Fraction 8	0.5	5.5	3.3	-
Fraction 9	0.5	5.0	3.1	-
Fraction 10	0.5	1.6	.9	-
Fraction 11	0.5	1.5	-	-
Fraction 12	0.5	1.3	-	-
Fraction 13	0.5	.75	-	-
Fraction 14	0.5	.5	-	-
Fraction 15	0.5	.5	-	-
Fraction 16	0.5	.34	-	-
Fraction 17	5	.39	-	-
Fraction 18	5	.26	-	-
Fraction 19	5	.15	-	-
Fraction 20	5	.10	-	-
Fraction 21	5	.05	-	-
Fraction 22	28	.013	-	-

[a] 25,000 rpm for 6 hours at 18°C using SW-25.2 rotor.

[b] A total of 165 x 10^5 PFU or 15.3% of the input was recovered from this experiment.

[c] Values shown for % yield were calculated from the total recovered PFU.

[d] 45 ml virus sample layered over 6 ml of CsCl with a density of 1.27.

Table 15. Density gradient centrifugation of partially purified HCV into cesium chloride[a]

Sample	Fraction vol. (ml)	Total PFU x 10^5[b]	% yield[c]	Density[d]
Virus control	50	750	(100)	--
Fraction 1	3	34.8	8.1	1.174
Fraction 2	3	360	83.5	1.128
Fraction 3	1	14.0	3.5	1.105
Fraction 4	1	2.9	.7	<1.1
Fraction 5	1	1.5	.4	<1.1
Fraction 6	1	1.5	.4	<1.1
Fraction 7	1	.55	.1	<1.1
Fraction 8	5	2.95	.7	<1.1
Fraction 9	5	1.65	.4	<1.1
Fraction 10	5	2.6	.6	<1.1
Fraction 11	15	6.3	1.5	<1.1
Fraction 12	5	2.7	.6	<1.1

[a] 25,000 rpm for 6 hours at 18°C using SW-25.2 rotor.

[b] A total of 431.4 x 10^5 PFU or 58% of the input was recovered.

[c] Values for % yield were calculated from the total recovered PFU.

[d] 50 ml virus sample layered over 6 ml CsCl with a density of 1.27.

Table 16. Density gradient centrifugation of partially purified HCV into cesium chloride[a]

Sample	Fraction vol. (ml)	Total PFU x 10^5[b]	% yield[c]	Density[d]
Virus control	50	400	(100)	--
Fraction 1	3	.03	.01	1.31
Fraction 2	3	.15	.07	1.26
Fraction 3	1	.93	.47	1.21
Fraction 4	1	8.2	4.16	1.19
Fraction 5	1	65	33.0	1.163
Fraction 6	1	115	58.5	1.135
Fraction 7	1	3.6	1.8	1.11
Fraction 8	5	1.8	.9	<1.10
Fraction 9	5	.45	.23	<1.10
Fraction 10	5	.30	.15	<1.10
Fraction 11	15	.35	.18	<1.10
Fraction 12	15	.95	.48	<1.10

[a] 25,000 rpm for 6 hours at 18°C using SW-25.2 rotor.

[b] A total of 196.8 x 10^5 PFU or 49% of input was recovered.

[c] Values for % yield were calculated from the total recovered PFU.

[d] 50 ml of virus layered over 6 ml of CsCl with a density of 1.50.

Table 17. Density gradient centrifugation of partially purified HCV into sucrose[a]

Sample	Fraction vol. (ml)	Total PFU x 10^5[b]	% yield[c]	Density[d]
Virus control	50	300	(100)	--
Fraction 1	3	.015	.01	1.288
Fraction 2	3	.36	.13	1.274
Fraction 3	2	189	72	1.175
Fraction 4	2	44.2	17	<1.10
Fraction 5	2	2.2	.8	<1.10
Fraction 6	2	.46	.14	<1.10
Fraction 7	2	.24	.09	<1.10
Fraction 8	22	3.0	1.1	<1.10
Fraction 9	20	1.4	.54	<1.10

[a] 25,000 rpm for 6 hours at 18°C using SW-25.2 rotor.

[b] A total of 261 x 10^5 PFU or 87% of input was recovered.

[c] Values for % yield were calculated from the total recovered PFU.

[d] 50 ml of virus layered over 9 ml of sucrose wih a density of 1.286.

At the conclusion of each centrifugal run, the tubes were inspected for the presence and location of visible zones of concentrated material. Three distinct zones of opalescence were observed in tubes at the end of each rate-zonal centrifugation experiment. In general, two rather broad, lightly colored bands of opalescence were separated by a single, narrow, orange colored band. In cesium chloride, the orange colored band was always associated with the highest infectivity titers found in the tube. Also in cesium chloride, the uppermost opalescent band appeared to be stratified. However, in the sucrose gradient the three bands appeared to be narrower, more distinct but closer together. A more important difference between the two density mediums regards the location of fractions with peak infectivity with respect to the orange colored band. In the sucrose gradient, the orange colored material was collected in Fraction 4 (Table 17) while the bulk of infectivity was found in Fraction 3.

Another notable feature of all four experiments was the density of fractions containing the peak infectivity titers. In the cesium chloride experiments, the density of such fractions was 1.13-1.14 (Tables 14-16), while with sucrose this value was 1.175 (Table 17). Since cesium chloride evidently produced a less abrupt gradient than sucrose, the "zoning" of HCV in regions with densities of about 1.14 is easily recognized from the data. It is also apparent from the data that the duration of centrifugation was not sufficient to

allow complete "banding" of infectivity. This is evidenced by the "tailing-off" of infectivity from the peak fraction into regions of lower densities. The abrupt decrease of infectivity between the peak fraction and fractions of higher densities suggested a buoyant density of about 1.14 Gm/ml for HCV in cesium chloride.

Isopycnic Density Centrifugation of HCV in Cesium Chloride and Sucrose

Isopycnic density gradient centrifugation of HCV was conducted primarily to obtain a high degree of purity and concentration required for electron microscope studies. It would also provide buoyant density data for comparison with findings published by Horzinek (37).

Two isopycnic centrifugations were conducted in cesium chloride, while a third was in sucrose. The results of these experiments are presented in Tables 18, 19, and 20.

The two successful experiments (Tables 18-19) clearly demonstrate very sharp infectivity peaks between densities of 1.140 and 1.150. These values represent the buoyant density of the HCV used in this study. Figures 3 and 4 graphically illustrate these data as functions of the % PFU in each fraction and the fraction density.

Failure of the isopycnic experiment in sucrose (Table 20) was an unexpected event, especially after rate-zonal centrifugation resulted in higher infectivity yields than in

Table 18. Isopycnic density centrifugation of partially purified concentrated HCV in cesium chloride[a]

Sample	Fraction vol. (ml)	Total PFU x 10^5[b]	% yield[c]	Density
Virus control	3.6	430	(100)	(1.15)
Fraction 1	0.5	<.01	<.1	1.225
Fraction 2	0.5	<.01	<.1	1.200
Fraction 3	0.5	.52	.45	1.189
Fraction 4	0.5	3.26	2.9	1.169
Fraction 5	0.5	3.63	3.2	1.156
Fraction 6	0.5	36.3	32	1.148
Fraction 7	0.5	60.0	52.5	1.140
Fraction 8	0.5	5.9	5.2	1.131
Fraction 9	0.5	.55	.48	1.125
Fraction 10	0.5	4.03	3.5	1.117

[a] 39,000 rpm for 24 hours at 18°C using SW-39L rotor.

[b] A total of 114.2 x 10^5 PFU or 27% of input was recovered.

[c] Values for % yield were calculated from the total recovered PFU.

Table 19. Isopycnic density centrifugation of partially purified-concentrated HCV in cesium chloride[a]

Sample	Fraction vol. (ml)	Total PFU x 10^5[b]	% yield[c]	Density
Virus control[d]	3.5	18.2	(100)	--
Virus control (CsCl)[d]	0.5	3.05	--	1.149
Pellet	0.5	<.00025	<.01	--
Fraction 1	0.5	<.00025	<.01	1.217
Fraction 2	0.5	<.00025	<.01	1.179
Fraction 3	0.5	.075	1.8	1.160
Fraction 4	0.5	4.05	94	1.148
Fraction 5	0.5	.175	4.1	1.135
Fraction 6	0.5	.00025	<.01	1.128
Fraction 7	0.5	<.00025	<.01	1.118
Fraction 8	0.5	<.00025	<.01	1.113
Fraction 9	0.5	<.00025	<.01	1.109
Fraction 10	0.5	<.00025	<.01	1.108

[a] 39,000 rpm for 24 hours at 18°C using the SW-39L rotor.

[b] A total of 4.3×10^5 PFU or 24% of input was recovered.

[c] Values for % yield were calculated from the total recovered PFU.

[d] Virus control indicates titer of HCV just prior to mixing with CsCl. Virus control (CsCl) indicates HCV titer in CsCl (density = 1.148) after 24 hours at 4°C.

Table 20. Isopycnic density centrifugation of partially purified-concentrated HCV in sucrose[a]

Sample	Fraction vol. (ml)	Total PFU x 10^5	% yield	Density
Virus control[b]	2.3	6.9	(100)	(1.15)
Pellet[c]	0.5	<.0005	-	-
Fraction 1	0.5	<.0005	-	1.163
Fraction 2	0.5	<.0005	-	1.151
Fraction 3	0.5	<.0005	-	1.150
Fraction 4	0.5	<.005	-	1.150
Fraction 5	0.5	<.005	-	1.149
Fraction 6	0.5	<.0005	-	1.148
Fraction 7	0.5	<.0005	-	1.145
Fraction 8	0.5	<.0005	-	1.143
Fraction 9	0.5	<.0005	-	1.138

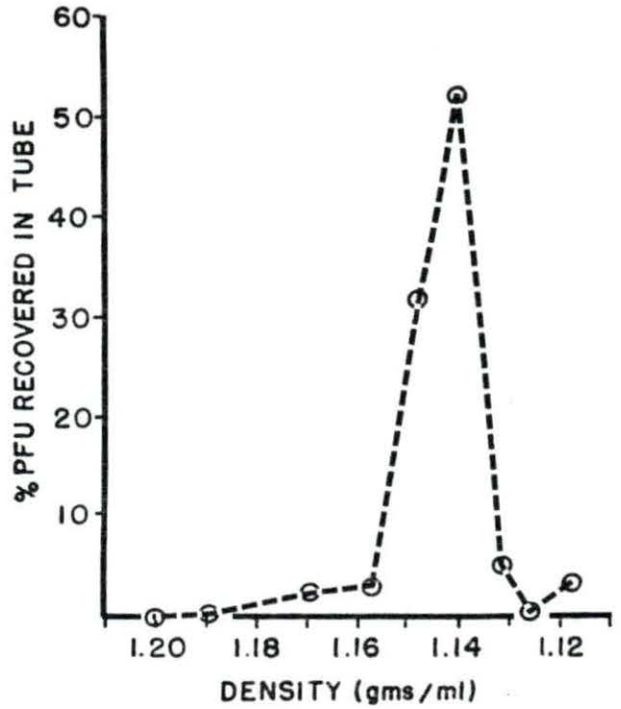
[a]39,000 rpm for 48 hours at 10°C using SW-39L rotor.

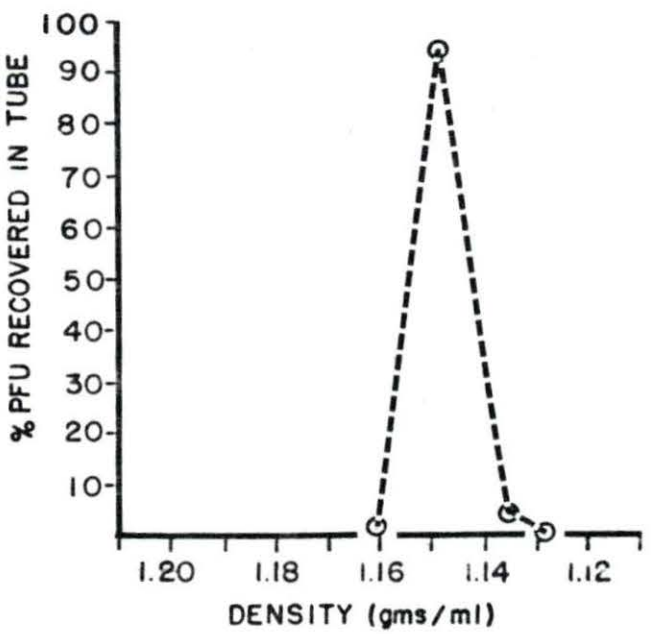
[b]Another virus control in sucrose at a density of 1.150 contained no detectable infectious HCV.

[c]Pellet material resuspended in 0.5 ml 0.01M TBS.

Figure 3. Isopycnic density centrifugation of HCV in cesium chloride. Buoyant density of HCV indicated by infectivity peak at 1.14 Gm/ml. Data from Table 18.

Figure 4. Isopycnic density centrifugation of HCV in cesium chloride. Buoyant density of HCV indicated by infectivity peak at 1.148 Gm/ml. Data from Table 19.


60
50
40
30
20
10
% PFU RECOVERED IN TUBE
1.20
1.18
1.16
1.14
1.12
DENSITY (gms/ml)


100
90
80
70
60
50
40
30
20
10
% PFU RECOVERED IN TUBE
1.20
1.18
1.16
1.14
1.12
DENSITY (gms/ml)

cesium chloride. Possibly, the sudden mixing of the dense sucrose medium and virus just prior to centrifugation produces an osmotic shock sufficient to destroy the virus.

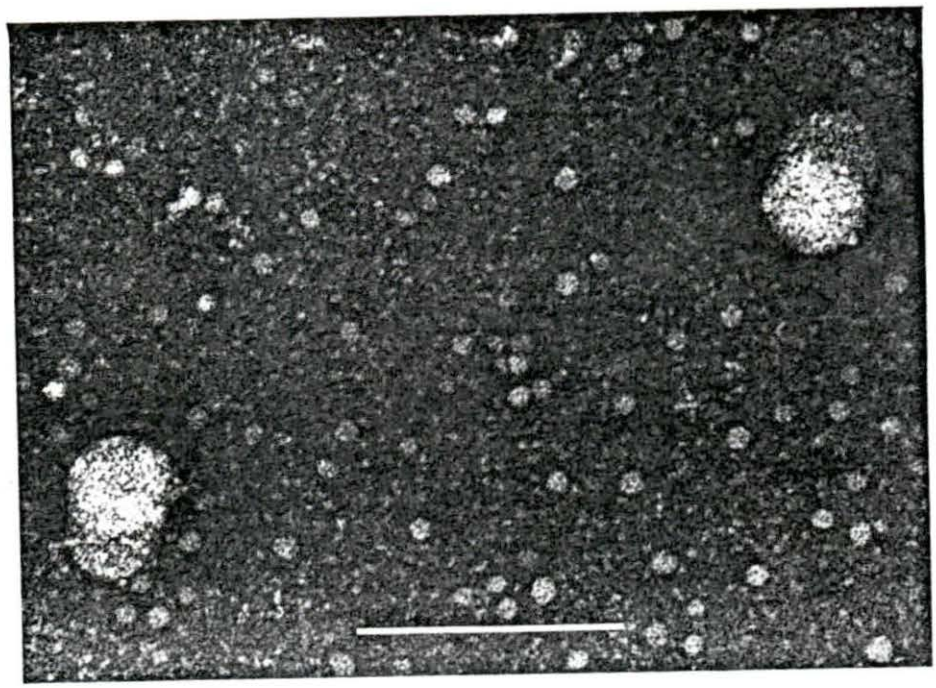
No zones of opalescence or other evidence of stratification were observed after isopycnic centrifugation in either cesium chloride or sucrose. After one experiment in cesium chloride there was a slight pellicle which suggested flotation of lipid material. Also, a barely visible quantity of clear gelatinous material was pelleted in each isopycnic experiment.

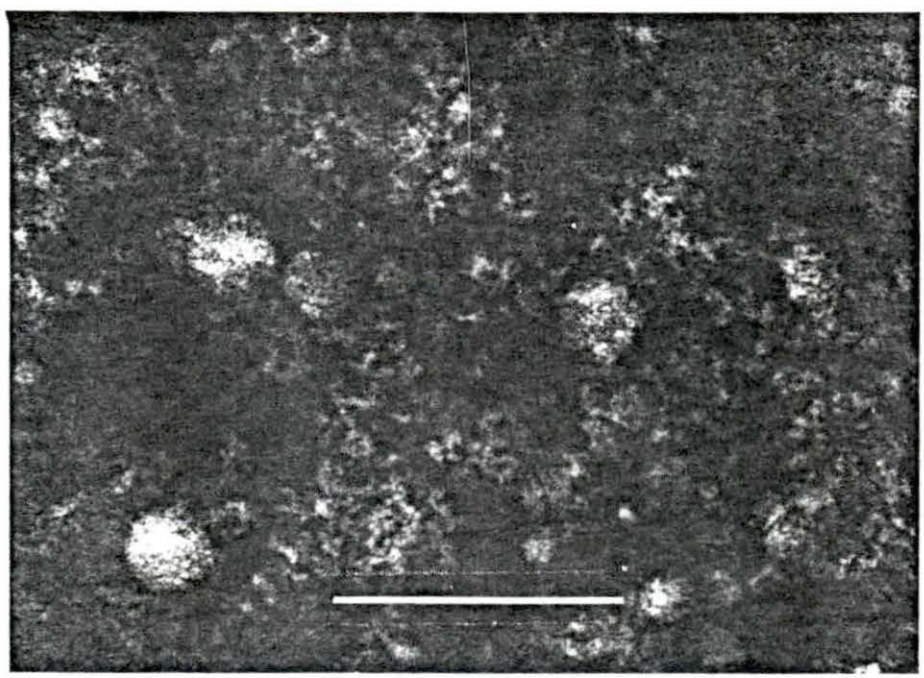
Electron Microscopy of HCV Preparations

As previously described, the pellet material and the isopycnic fraction with the highest concentration of infective HCV (Table 18, Fraction 7) were examined with the electron microscope. The characteristic virus-like particles observed during these examinations are illustrated in Figures 5 and 6. Figure 5 illustrates two of the largest characteristic particles found in isopycnic fractions with peak infectivity titers. A variety of smaller particles also observed in such samples appear in Figure 5, the most prominent of which are 12-15 mμ in diameter. Although somewhat asymmetric, the largest characteristic particles are 40-50 mμ in diameter and are surrounded by a sac-like appendage or membrane. Figure 6 illustrates several characteristic 40-50 mμ particles similar

Figure 5. Electron micrograph of characteristic 40-50 mμ virus-like particles from isopycnic fractions (Table 18) with peak infectivity. Note asymmetric arrangement of sac-like membrane surrounding each particle. Also, note large number of 12-15 mμ particles. Bar = 100 mμ. (Magnified x 350,000)

Figure 6. Electron micrograph of 40-50 mμ virus-like particles and 12-15 mμ entities aggregated by HCV antibody. Original sample was an aliquot of material used for Figure 5. Bar = 100 mμ. (Magnified x 380,000)





to those in Figure 5 except that they are incorporated in an amorphous mass of aggregated material. Such aggregates were found in an aliquot of the high titer isopycnic fraction after interaction with dilute FITC-conjugated antibody #7802 overnight at 4°C. The more distinct 40-50 mμ particles in Figure 6 have a regular spherical to hexagonal symmetry but there is no evidence for an enveloping membrane.

During a comparable scanning period with pellet material, no particles similar to those illustrated in Figure 5 were observed. FITC-conjugated antibody thus was not used with the pellet material.

DISCUSSION

The results of this study indicate that HCV may be partially purified using a simple batch-type chromatographic procedure with MFO. The virus is avidly adsorbed to MFO from isotonic solutions and is eluted by solutions with low ionic strength at a pH near 9.7. This is in contrast to most chromatographic procedures where an increase in ionic strength is customarily used to effect elution. Although there was some variability in the data, generally 50 to 75% of the infectious HCV was recovered when MFO-HCV complexes were dissociated with 0.01M aqueous NaCN. Approximately 90-95% of the extraneous organic nitrogen was removed with this procedure.

It was also observed that 0.003M NH_4OH in water (pH 9.7) was at least as efficient as 0.01M NaCN for dissociating MFO-HCV complexes. Although it was not possible to extensively investigate the NH_4OH eluting solution, its use may have a distinct advantage over the cyanide solution. That is, it may be possible to obviate the 18-24 hour dialysis procedure required to remove cyanide ions, by neutralizing the NH_4OH with a weak acid and adjusting the solution to isotonicity with a concentrated saline-buffer solution. Thus, the MFO-purified HCV would be exposed to low ionic strength, and relatively high pH for a period of 10-20 minutes rather than several hours.

Pretreatment of MFO with 0.001M NaCN substantially reduces the amount of colloidal ferric oxide generated during the elution procedure and also affected its binding capacity for HCV, when compared to non-pretreated MFO. This was evidenced by greater quantities of HCV in the MFO-supernates and pooled washings, and recovery of most of the adsorbed HCV in the first eluates. The reasons for this change in adsorption behavior are unknown. However, the fact that pretreated MFO settles out with normal gravity somewhat slower and has a larger packed volume than non-pretreated MFO, suggests an increased hydration shell around pretreated MFO particles.

Evidence is presented that the total nitrogen of a virus sample has no reliable correlation with the amount of MFO needed to adsorb all the virus in the sample. This is indicated by the failure of a sequential readsorption and elution procedure applied to MFO-purified HCV as a concentration step (Table 10). These data suggest that MFO selectively adsorbs a certain class of proteins which happens to include HCV. Also, the fact that HCV contains essential lipids (10, 11, 12), and MFO adsorbs other lipo-viruses (39), suggests that the lipo-protein component of HCV is the substance which binds to MFO.

Another interesting observation made during this study regards the progressive increase of adsorption capacity of MFO after sterilization. It should be noted that this phen-

omenon was not recognized until the first batch of MFO was several weeks old. Although no systematic study of this phenomenon was made the second and third MFO batches were tested within hours after sterilization. This provided data (Table 9) which indicates that the progressive increase of adsorption capacity commences immediately after sterilization in the autoclave. Although the reasons for this behavior are unknown, one may speculate that the heat and pressure applied during sterilization and the subsequent de-gassing upon cooling and depressurization in some way affects the hydration of MFO particles. It is possible that a small increase in the hydration of MFO particles could result in a substantial decrease in its adsorption capacity as a consequence of the increased distance over which binding energies must act. Investigation of this phenomenon quite possibly would provide much information regarding the nature of the binding energies between MFO and lipo-proteins as well as additional physical-chemical data for HCV. In this respect it is significant that influenza viruses are eluted from MFO by saturated phosphate or carbonate solutions (39) while HCV was eluted only by solutions with low ionic strength and a relatively high pH. Thus, two lipo-viruses are eluted under vastly different ionic conditions indicating that both viruses possess unique physical-chemical surface properties relative to each other.

Although this study involved only a batch-type procedure with MFO, its use in a column might provide a more definitive means for investigating the chromatography of HCV or lipo-

proteins. Since MFO particles would pack too tightly in a column for practical flow rates, it would be necessary to prepare columns with an MFO-coated carrier particle such as #40 mesh iron filings or small plastic-coated magnetic beads. The iron filings could be magnetized by using the iron column itself as the core of an electro-magnet surrounded by a coil connected to a direct current source.

Since MFO particles are non-toxic to cultured cells (48) and are recognized and ingested as foreign bodies by macrophages, it would be interesting to investigate the immunologic adjuvant potential of MFO. Results of the present study indicate that the isotonic environment found in vivo would prevent dissociation of MFO-HCV complexes. Thus, such complexes might be rapidly ingested by macrophages and transported to regional lymph nodes or other areas of antibody synthesis. Also, the intracellular location of the MFO-bound antigen would preclude inactivation by antibody during transport to the sites of antibody synthesis.

Another consideration suggested by results of the present study regards the possible agglutinations of MFO-HCV complexes by specific antiserum. Such a phenomenon could be modified as a simple diagnostic test for HCV as well as for titrations of HCV and its antibody. However, such an investigation would require use of non-magnetic ferric oxide since MFO auto-agglutinates in aqueous suspension due to its magnetism.

Finally, the prolonged exposure of HCV to the cyanide, EDTA and saturated phosphate and carbonate solutions described in this study (Table 2) resulted in the loss of no more than 50% of the infective HCV. This observation suggests that with care, HCV may be relatively stable to vigorous chemical treatment; a fact which constitutes an encouraging note to future chemical-physical studies with this virus.

Results of rate-zonal density gradient experiments conducted to concentrate HCV indicate that the 6-hour duration of centrifugation was sufficient to pack the virus into relatively narrow bands of concentrated infectivity. The "tailing-off" of infectivity indicates that slightly longer durations of centrifugation would have resulted in somewhat narrower bands of concentrated virus.

Some variability of data from rate-zonal experiments in cesium chloride regarding the volume and location of the bands of concentrated HCV is indicated in Tables 14, 15 and 16. This may be explained by the fact that both the initial density of cesium chloride and the number and volume of fractions collected were also variable. Results of the most successful experiment (Table 15) show that a high yield of recovered virus was obtained in a single 3 ml sample when the initial density of cesium chloride was 1.27. These results indicate that additional experiments might have determined an optimal procedure for cesium chloride regarding the initial density and the number and volume of fractions to be collected.

The fact that sucrose diffuses rather slowly in 0.01M TBS relative to cesium chloride is evidenced by the steep density gradient or "shelf" found at the end of the rate-zonal

experiment in sucrose (Table 17). As a result of this steep gradient, the bulk of HCV infectivity banded into a fraction volume probably no larger than 2 ml. Thus, it would appear that rate-zonal experiments in sucrose might provide narrower density bands for the concentration of HCV. In addition, the recovery of 87% of the infective virus in the sucrose experiment emphasizes the advantage of using sucrose rather than cesium chloride as a density medium for concentrating HCV.

As previously indicated, the fact that concentrated bands of HCV in the rate-zonal experiments with cesium chloride occurred at densities between about 1.13 and 1.14, suggested that the buoyant density of the virus is in this region. However, the data presented in Table 17 suggests that HCV may have a buoyant density in sucrose of about 1.17-1.18. This discrepancy of density values may be explained by the abruptness of the density gradient produced by sucrose. Thus, Fraction 3, Table 17 probably was a mixture of densities both higher and lower than 1.175 with HCV concentrated in one of the lower densities.

The two successful isopycnic density experiments in cesium chloride indicate that the buoyant density of the strain of HCV used in this study is between 1.14 and 1.15 Gm/ml. This finding shows good agreement with the 1.16 Gm/ml for the HCV strain used by Horzinek (37). However, there was no evidence in the present study which suggested the secondary infectivity peak in isopycnic experiments indicated by Horzinek's report.

Perhaps her findings regarding the primary and secondary infectivity peaks and the slightly higher buoyant density value might be due to the fact that her experiments were conducted using relatively crude HCV preparations; i.e., a fluid-phase, cell-cultured virus containing 10% fetal calf serum. Thus, the secondary peak may have been due to involvement of virus with sub-cellular components, while the slight difference in buoyant density may be a result of simple differences in experimental technique and the strains of HCV used. The results of the present isopycnic studies are interpreted as essentially verifying the buoyant density for HCV determined by Horzinek.

Since about 75% of the HCV infectivity was lost during the isopycnic experiments in cesium chloride, the question might be raised whether any of the surviving infectivity represents virus which had sustained some damage due to partial degradation. However, since even partial degradation would probably increase the buoyant density of HCV with the result that one or more lesser infectivity peaks would be found at densities higher than those obtained in this study. Although there is a suggestion of this effect in Figure 3, most of the recovered infectivity was found at densities that essentially agree with Horzinek's findings. Thus, the buoyant densities determined for HCV in the present study are interpreted as applying to intact virus.

Loss of virtually all infectivity during the isopycnic experiment in sucrose (Table 20) might be explained by osmotic shock produced by the sudden mixing of 60% sucrose and HCV. Although the molarity of cesium chloride and sucrose are very similar at a density of 1.15 Gm/ml, the production of two ions by cesium chloride in solution indicates that its osmotic pressure should be about twice that of sucrose. Thus, the concept regarding osmotic shock with sucrose was at first overlooked. However, since it is known that cesium chloride diffuses much more rapidly than sucrose, it is quite possible that the sucrose solution caused a destructive dehydration of HCV before concentration differences could be equilibrated by diffusion. The superior results obtained with the rate-zonal experiments in sucrose may be explained then as due to a gradual penetration of HCV into the sucrose density gradient. Since sucrose is a far more economic density medium compared to cesium chloride, it would be advantageous to study the buoyant density of HCV in sucrose using methods, such as dialysis, which gradually increase density to the desired level. Since HCV has an unusually low buoyant density, other substances with low densities, such as glycerol, might be considered for use in isopycnic experiments with this virus.

Use of refractometry for rapidly determining the densities of rather large numbers of samples without substantial loss of sample volume was of significant benefit in this study. The

relationship between refractive index and density has been discussed by Ifft et al. (49) and applied to buoyant density investigations of HCV by Horzinek (37). The linear relationship between density and refractive index for cesium chloride and sucrose further simplified determination of density by refractometry. Therefore, the density of unknown samples may be obtained simply by consulting an appropriate standard plot, prepared by determining refractive indices for weighed samples of the density medium being used.

The electron micrographs of concentrated, MFO-purified HCV (Figures 5 and 6) present evidence that the virus has a spherical to hexagonal symmetry, with a diameter of about 40-50 mμ, and is surrounded by a poorly defined sac-like membrane. The estimated size of 40-50 mμ disagrees with earlier estimates of 15-35 mμ (4, 5, 6, 7, 8), most of which were based on calculations derived from filtration experiments with membranes of graded porosity. It is important to emphasize at this point that the presence and fate of HCV throughout the procedures described in this study were indicated by direct infectivity assays which required interaction with FITC-conjugated homologous antibody. Thus, electron micrographs were made from a purified-concentrated sample of infectious HCV and furthermore no virus-like entities were observed in the sample other than those illustrated by Figures 5 and 6. In addition, the 40-50 mμ particles were observed to be involved in aggregated masses after interaction with homologous antibody

(Figure 6). It is therefore concluded that the 40-50 mμ particles illustrated in Figure 5 are images of infective HCV.

The nature of the 12-15 mμ entities illustrated in Figure 5 is at present only speculative. Since their presence in great numbers suggests that their buoyant density must be similar to that of HCV, it is conceivable that they may represent a degradative product of HCV; i.e., "soluble antigen" (36). However, it should be noted that such degradation could have occurred during dialysis against distilled water after isopycnic centrifugation, prior to electron microscopy. Thus, there would be no need for assuming a buoyant density similar to HCV. The fact that the 12-15 mμ particle also appeared to be involved in the aggregated masses illustrated by Figure 6 further suggests a possible relation to HCV. Obviously, the nature and identity of such particles requires further study.

Perhaps the most striking recent electron micrographs of HCV material were those published by Mayr and Mahnel (26) showing both adenovirus particles and smaller picornavirus-like particles 22 mμ in diameter. The HCV used by these workers produced cytopathic effects in cell cultures which could be neutralized by hog cholera antiserum. Although no decision was made concerning the identity of the small virus particles in the electron micrographs reported by Mayr and Mahnel, their 22 mμ diameter is consistent with the earlier size estimates for HCV. However, in addition to the morpho-

logical resemblance to the picornaviruses as suggested by Mayr and Mahnel, the 22 mμ virus entity bears a striking resemblance to the DNA-type chloroform resistant "satellite" viruses frequently associated with adenoviruses. The physical, biological and immunological characteristics of the newly recognized "satellite" or adeno-associated viruses have been described by Hogan et al. (50). Thus, the 22 mμ particle described by Mayr and Mahnel should be investigated with respect to its nucleic acid type and its stability to chloroform before being considered as a possible HCV particle.

SUMMARY

Development of a batch-type chromatographic procedure with magnetic ferric oxide (MFO) to partially purify cell-cultured hog cholera virus (HCV) is described. The findings indicate that MFO adsorbed infectious HCV from isotonic solutions and was subsequently eluted under conditions of low ionic strength and relatively high pH (ca. 9.7). Dilute solutions of sodium cyanide (0.01M) and ammonium hydroxide (0.003M) effectively dissociated MFO-HCV complexes. Evidence is presented that 50-75% of the original HCV infectivity can be recovered with such a procedure concomitant with a 90-95% reduction of extraneous organic nitrogen.

MFO-purified HCV was concentrated by rate-zonal type density gradient centrifugation in buffered solutions of cesium chloride and sucrose. There is evidence that HCV is most stable in the buffered sucrose solution. Concentrated MFO-purified HCV was subjected to isopycnic gradient experiments in cesium chloride and sucrose. As a result of procedures used to prepare HCV for isopycnic centrifugation in sucrose, all infectivity was lost. However, two successful isopycnic centrifugations of HCV in cesium chloride indicated a buoyant density of 1.14-1.15 Gm/ml for the virus.

The isopycnic density fraction containing the highest HCV infectivity titer was negatively stained with phosphotungstic acid and examined with an electron microscope.

Electron micrographs were obtained which show characteristic virus-like particles 40-50 mμ in diameter and large numbers of unidentified 12-15 mμ entities. The 40-50 mμ particles were surrounded by a poorly defined sac-like membrane. Both particles were aggregated by interaction with specific HCV antibody. In the antibody-aggregated masses the 40-50 mμ particle had spherical to hexagonal symmetry but visual evidence of the membrane was obscured. Although it is most likely that the 12-15 mμ entity is a degradation product of the 40-50 mμ particle, its true identity remains speculative. However, it is concluded that the enveloped 40-50 mμ particle is an image of the intact, infective form of HCV.

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